# Studies of genome-wide patterns of gene expression and methylation in osteosarcoma

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## ABBREVIATIONS

| | |
|---|---|
| 3' UTR | 3 prime untranslated region |
| 5' UTR | 5 prime untranslated region |
| CGH | Comparative genomic hybridization |
| CGI | CpG island |
| CLL | chronic lymphocytic leukemia |
| CpG | CG dinucleotides |
| DNA | Deoxyribonucleic acid |
| DNMT | DNA methyltransferase |
| FDA | the US Food and Drug Administration |
| HDAC | histone deacetylase |
| kb | kilobase |
| LOH | loss of heterozygosity |
| LOI | loss of imprinting |
| mRNA | Messenger RNA |
| miRISC | miRNA-induced silencing complex |
| miRNA | microRNAs |
| MSP | methylation specific PCR |
| PBA | 4-phenylbutyric acid |
| PCR | polymerase chain reaction |
| qMSP | Quantitative methylation-specific polymerase chain reaction |
| qRT-PCR | Quantitative real-time reverse-transcription PCR |
| pre-miRNA | precurson miRNA |
| RNA | ribonucleic acid |
| RNAi | RNA interference |
| SAHA | suberoylanilide hydroxamic acid |
| SNP | single nucleotide polymorphism |
| TSS | transcription start site |
| WHO | World Health Organization |

*Gene symbols*

| | |
|---|---|
| AGO | Argonaute |
| CDKN2A | cyclin-dependent kinase inhibitor 2A |
| CXCL12 | ligand stromal-cell-derived factor 1a |
| CXCR4 | chemokine (C-X-C motif) receptor |
| GW182 | glycine-tryptophan protein of 182 kDa |
| MEST | mesoderm specific transcript homolog (mouse |
| MYC | v-myc myelocytomatosis viral oncogene homologue (avian) |
| PTEN | phosphatase and tensin homolog |
| RB1 | retinoblastoma 1 |
| RUNX2 | runt-related transcription factor 2 |
| TP53 | tumour protein p53 |

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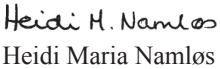
Oslo, 20 December 2012



Heidi Maria Namløs

## AIMS OF THE STUDY

In this thesis, genome-wide patterns of gene expression at the miRNA and mRNA level as well as DNA methylation in osteosarcoma have been studied. The aims for the papers were:

**Paper I:** Identification of metastatic signatures by gene expression profiling of primary and metastatic samples in osteosarcoma

**PaperII:** Identify miRNAs and putative mRNA target genes that are deregulated in osteosarcoma.

**Paper III:** Identify miRNAs that may be regulated by DNA methylation in osteoarcoma, and elucidate if the miRNAs play a role in the tumourigenic process.

## LIST OF PAPERS

**Namlos HM**, Kresse SH, Muller CR, Henriksen J, Holdhus R, Saeter G, Bruland OS, Bjerkehagen B, Steen VM, Myklebost O (2012) Global gene expression profiling of human osteosarcomas reveals metastasis-associated chemokine pattern. *Sarcoma* **2012:** 639038

**Namløs HM,** Meza-Zepeda LA, Barøy T, Østensen IHG, Kresse SH, Kuijjer ML, Serra M, Bürger H, Cleton-Jansen A-M, Myklebost O (2012) Modulation of the Osteosarcoma Expression Phenotype by MicroRNAs. *PloS one* **7:** e48086

**Namløs HM**, Skårn M, Ahmed D, Lind GE, Kresse SH, Serra, M. Llombart-Bosch A, Myklebost O, Meza-Zepeda LA. Epigenetic regulation of miRNA in osteosarcoma; an integrated analysis of genome-wide miRNA and DNA methylation changes. *Manuscript*.

**Related papers not included in the thesis**

Francis P, **Namlos HM**, Muller C, Eden P, Fernebro J, Berner JM, Bjerkehagen B, Akerman M, Bendahl PO, Isinger A, Rydholm A, Myklebost O, Nilbert M (2007) Diagnostic and prognostic gene expression signatures in 177 soft tissue sarcomas: hypoxia-induced transcription profile signifies metastatic potential. *BMC genomics* **8:** 73

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Kuijjer ML, **Namlos HM**, Hauben EI, Machado I, Kresse SH, Serra M, Llombart-Bosch A, Hogendoorn PC, Meza-Zepeda LA, Myklebost O, Cleton-Jansen AM (2011) mRNA expression profiles of primary high-grade central osteosarcoma are preserved in cell lines and xenografts. *BMC medical genomics* **4:** 66

Skarn M, **Namlos HM**, Noordhuis P, Wang MY, Meza-Zepeda LA, Myklebost O (2012) Adipocyte differentiation of human bone marrow-derived stromal cells is modulated by microRNA-155, microRNA-221, and microRNA-222. *Stem cells and development* **21:** 873-83

# INTRODUCTION

## An introduction to cancer

Cancer is a leading cause of death worldwide, accounting for around 13% of all deaths in 2008. Cancer is a group of more than 100 distinctive diseases, but 50% of all cancers worldwide are accounted by a few major types, in descending order, breast, colorectal, cervix uteri and lung cancer for women and lung, prostate, colorectal and stomach cancer for men. Looking at a mortality perspective, liver cancer has by far a lower incidence rate than prostate cancer, but causes more cancer deaths. About 70% of all cancer deaths occurred in low- and middle-income countries. Tobacco and alcohol use, unhealthy diet and physical inactivity are the main cancer risk factors worldwide, as well as chronic viral infections from hepatitis B and C and human papilloma virus causing liver and cervical cancer mainly in low-income countries (GLOBOCAN (Ferlay J et al. 2008)).

In Norway, the four most common cancer types are prostate, breast, colon and lung cancer, accounting for about 50% of all cancer cases. The incidence rates of cancer are increasing, reflecting an increased risk as well as an increasing ability to diagnose a number of cancer types. However, the survival rate continues to increase. This is in large part a result of increased attention regarding cancer from the patient and the health care system and from screening in the population, but is also linked to increased quality of treatment. The occurrence of cancer increases as individuals age, believed to be due to the time required to accumulate the genetic and epigenetic changes necessary for the onset of cancer. In Norway, 85% of men and 75% of women diagnosed with cancer are above the age of 55 (Cancer registry of Norway) (www.kreftregisteret.no/en/). Thus, the increase in number of cancers cases can partly be explained by an elderly population and increasing lifespan.

### Characteristics of cancer

Cancer is a genetic disease at the cellular level (Vogelstein and Kinzler 2004), and is derived from normal cells whose genome has become altered or damaged to such an extent that the cells become transformed and begin to exhibit abnormal malignant properties. The two main characteristics of cancer are uncontrolled growth of the cells forming an abnormal mass of

cells in the human body and the ability of these cells to migrate from the primary site and spread to distant sites (metastasize).

Tumours are divided into two main types: benign and malignant depending on their growth pattern. A benign tumour resemble closely the normal tissue, and does not spread or invade surrounding tissue, and once it is removed, doesn't usually recur. A benign tumour is not considered a cancer. Cancers, being malignant tumours, can be only locally invasive, but can also have the ability to leave the primary tumour, enter the circulation and form a secondary tumour in another organ. Metastasis is the major cause of death of cancer patients (Boyle and Levin 2008).

Tumours result from the unchecked growth of cells which, to an extent, resemble the tissue of origin. The primary tissue layers during embryo development are the three germ layers ectoderm (outer layer), mesoderm (middle layer) and endoderm (inner layer) from which all organs and tissues in the body originate. Carcinoma, like carcinoma of the breast, colon, liver, lung, pancreas, prostate, or stomach, is the most common type of cancer in humans. Carcinoma begins in a tissue that lines the inner or outer surfaces of the body, and generally arises from cells originating from the endodermal or ectodermal germ layer during embryogenesis. A sarcoma arises from transformed cells derived from the mesenchymal germ layer, being malignant tumours made of cancerous bone, cartilage, fat, muscle or vascular tissues. Other main groups are leukaemia originating from haematopoietic tissue, lymphoma from lymphatic tissue, and glioma from brain tissue.

### Development of cancer

The initiation and development of cancer is a multistep process. The clonal evolution model was originally proposed by Nowell in 1976. It suggests that malignant tumours develop from a single progenitor cell (monoclonal) or multiple (polyclonal) subpopulations (Figure 1a) that accumulates genetic and epigenetic changes in a sequential manner, providing a Darwinian clonal selection and selective growth advantage (Nowell 1976). Although tumours may be monoclonal, there is a high degree of diversity between and within tumours as well as among cancer-bearing individuals. Variation between patients is often referred to as inter-tumour

heterogeneity and is classically recognized through different morphology types and expression subtypes. Yet, at the time of diagnosis, the majority of human tumours display both morphological and physiological intra-tumour heterogeneity, and there is strong evidence for the co-existence of genetically and epigenetically divergent tumour cell clones. All of these factors together determine the risk of disease progression and therapeutic resistance. Thus, different models are plausible for the explanation of intra-tumour heterogeneity (reviewed by (Russnes et al. 2011)).

In a second model, it is suggested that normal cells are derived from a lineage of stem cells that have the capacity to self-renew and differentiate into specialized cell types. A population of cells with stem-cell properties may thus be responsible for tumour formation (Reya et al. 2001). A major focus of cancer research is identifying these "cancer stem cells", cells with the ability to initiate and sustain the growth of a tumour. In contrast to the clonal evolution model, the cancer stem cell model suggests a hierarchical organisation in which tumour heterogeneity is explained by several rare precursor cells, each giving rise to a different subpopulation within the tumour (Figure 1b). A third model, the mutator hypothesis, suggests that tumours progress by the gradual and random accumulation of mutations as the tumour grows causing a high degree of diversity in the tumour (Figure 1c) (Loeb et al. 1974). As illustrated in Figure 1d, different progression models can result in distinct spatial distribution of subpopulations.

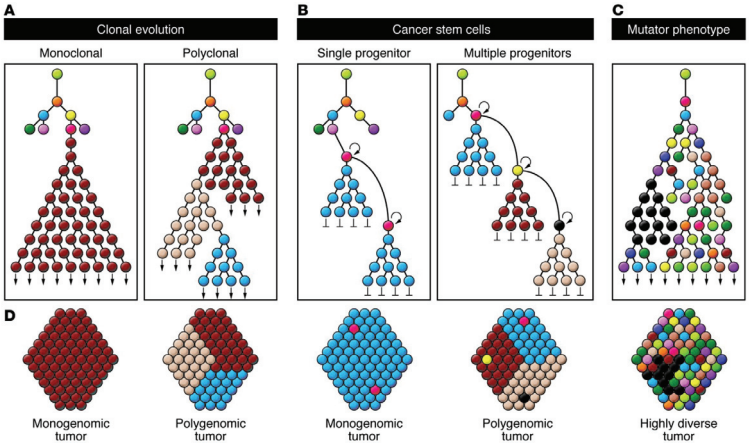


**Figure 1.** Hypothetical models explaining intratumour heterogeneity. (A–C) Different models of tumour progression can give rise to distinct types of intratumour heterogeneity, exemplified here by the clonal evolution (A), the cancer stem cell (B), and the mutator phenotype (C) models. (D) The different models can result in distinct spatial distributions of subpopulations Reprinted by permission from American Society for Clinical Investigation, The Journal of Clinical Investigations (Russnes et al. 2011).

During the development of normal cells to cancers a set of common traits (hallmarks) have been described to be deregulated, reducing the complexity of cancers to a more limited number of biological principles. The initial six hallmarks were; sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis (Hanahan and Weinberg 2000). In 2011, Weinberg and Hanahan proposed two new hallmarks; the capability to reprogram or modify cellular metabolism and to evade destruction by the immune system. These eight hallmarks of cancer are capabilities that allow cancer cells to survive, proliferate and disseminate. This is made possible through two underlying enabling characteristics; the development of genomic instability and mutations causing genetic changes, and secondly inflammation where cells of the immune system serve to promote tumour progression (Hanahan and Weinberg 2011).

## Metastasis

The process of metastasis consists of a long series of sequential, interrelated steps (Figure 2). Cancers have the ability to divide perpetually, are locally aggressive and penetrate normal body surfaces and barriers, and invade into surrounding tissues and organs. Cancers may also spread to other sites within the body by entering into the lymphatic and/or the blood vessels. If these circulating cancer cells manage to survive in the circulation and evade the host immune system, they may extravasate at a secondary organ site, adhere and survive in the new microenvironment and finally form neovasculature to allow growth at the target organ site. These metastases may then invade and damage nearby tissues (Steeg 2006). If this process of continuous growth is not halted, the body suffers a burden of tumour cells throughout the body interfering with normal functions, and death ultimately ensues.

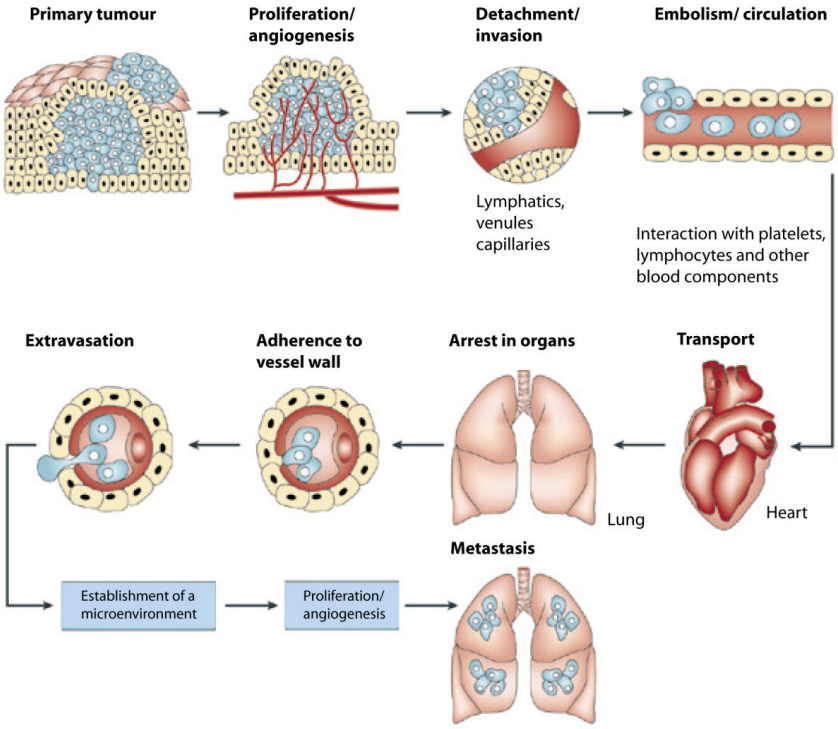


**Figure 2.** The metastatic process from primary tumour to the development of metastases. Reprinted by permission from Macmillan Publishers Ltd, Nature (Fidler 2003), copyright.

There is a propensity for certain tumours to seed in particular organs. In 1889, Stephen Paget put forward the hypothesis that metastasis depends on cross-talk between selected cancer cells (the 'seeds') with an affinity for specific organ microenvironments (the 'soil') (Paget 1989). In addition to the cancer cells, tumours contain stromal cells that are non-cancerous supporting and surrounding cells. Fibroblasts, immune cells, pericytes and inflammatory cells are the most common types of stromal cells. The microenvironment is believed to contribute substantially to malignancy, and cancer cells are able to alter their stromal environment, usually by producing stroma-modulating growth factors (Mueller and Fusenig 2004). Thus, after all these years, the theory of Paget still holds, and we know that the potential of a tumour cell to metastasize depends on its interactions with the factors that promote tumour cell growth, survival, angiogenesis, invasion and metastasis.

Each of the steps of the metastatic process can be rate limiting, and cancer-cell metastasis can be blocked at a variety of stages including deficiencies in invasion, survival and proliferation in the circulation and deficiencies that prohibit metastasis. Furthermore, susceptibility to attack by the immune system and an inability to grow at the final metastatic site may prelude the metastatic process. Current treatment strategies target the primary tumour rather than metastases. Targeting the metastatic features might enhance the efficacy of the treatment. Metastatic lesions may develop from a small number of cancer stem cells which can change shape and properties to disseminate into the organism and adapt to the conditions of different organs (Li et al. 2007). Identification of the cancer stem cells may lead to novel therapeutic strategies against metastatic cancer.

## Cancer as a genetic disease

Cancer is a genetic and epigenetic disease. The deregulated expression of genes and non–protein coding RNAs, which occur through both genetic mechanisms (*e.g*. mutation and copy number alterations) and epigenetic mechanisms (*e.g*. promoter methylation and histone modifications), are known to promote tumour development and progression. However, genetic and epigenetic mechanisms are not separate events in cancer; alterations in epigenetic mechanisms can lead to genetic mutations, and genetic mutations in epigenetic regulators lead to an altered epigenome (You and Jones 2012). Gene mutations that are inherited or have arised *de novo* in the germ line cells (sperm and eggs) may be passed from generation to generation. This damaged gene puts them at higher risk for cancer than most people. Somatic mutations are genetic alterations acquired by any of the cells in the body except the germ cells.

Gene mutations change the nucleotide sequence of a gene. A point mutation is a type of mutation that causes the replacement of a single base nucleotide with another nucleotide of the genetic material. The term point mutation also includes insertions or deletions of a single base pair. If the point mutation occurs in the protein coding region, this may change the tri-nucleotide codon that codes for an amino acid or affect a splice site, and these changes are divided into three functional categories. A silent mutation codes for the same amino acid with no effect on the function of the protein. A missense mutation codes for a different amino acid, and dependent on the chemical similarity of the introduced amino acid this may change the

function of the protein. A nonsense mutation converts an amino acid codon into a termination codon, which can truncate the protein, and if essential motifs of the protein are lost it may not be functional. The abolishment of a splicing site results in one or more introns remaining in mature mRNA and can also lead to the production of aberrant proteins.

Humans have 46 chromosomes; 22 pairs of autosomal chromosomes (chromosome 1-22) and one pair of sex chromosomes (XY in males and XX in females). Chromosomal aberrations are alterations of the DNA that results in an atypical number of chromosomes or a structural abnormality in one or more chromosomes. Chromosomal abberations may be either numerical or structural. A numerical disorder is called aneuploidy (an abnormal number of chromosomes) where the copy number of one or several of the 46 human chromosomes is changed. Structural abnormalities refer to aberrations in the linear organisation of the sequences within a chromosome such as deletions, amplifications, inversions and translocations. Structural abnormalities can be unbalanced or balanced. Deletions lead to decreased amount of chromosomal material, and may involve the loss of the whole or segments of a chromosome arm, one of the chromosomes/gene copies in a pair (heterozygous) or both chromosomes/gene copies (homozygous). Amplifications lead to increased amount of chromosomal material, and involve the amplification or gain of small chromosomal segments or larger chromosomal regions. The term copy number variation (CNV) refers to a structural variation involving both duplications and deletions of sequences that typically range from 1,000 base pairs to 5 megabases. Translocations are rearrangements of chromosomal segments to another region of the same chromosome (intrachromosomal) or transfer of a segment to another chromosome (interchromosomal). Translocations may alter the gene structure by fusing promoter elements from one gene to the intact coding region of another gene, causing a deregulated, but normal protein. Alternatively, the coding regions of two genes may be recombined, generating a fusion gene with a different function from the two fusion partners.

### Cancer genes

Cancer-critical mutations may affect genes categorized as proto-oncogenes, tumour suppressor genes or DNA repair genes. Oncogenes and tumour suppressor genes include both messenger RNAs (mRNAs), coding for proteins, and small regulatory microRNAs

(miRNAs). Mutations in these genes can affect net cell growth and can thereby confer a selective growth advantage to the mutant cell.

An oncogene is a gene that has the potential to cause cancer. Most oncogenes are mutated versions of normal genes called proto-oncogenes. Proto-oncogenes regulate appropriate cell growth and differentiation under normal conditions, and are often involved in signal transduction. Oncogenes are mutated in ways that render the gene constitutively active or active under conditions in which the wild-type gene is not. Oncogenes are typically dominant, and only one allelle of the gene is generally required for the oncogenic effect to occur (Vogelstein et al. 2004).

A tumour suppressor gene (also called anti-oncogene) is a gene whose function is to regulate cell cycle checkpoints or promote apoptosis. Loss-of-function of a tumour suppressor gene inactivates the tumour suppressor protein and leads to cell transformation and tumour growth. Inactivation may arise from missense or nonsense mutations, from deletions or insertions of various sizes, or from epigenetic silencing (Vogelstein et al. 2004). Mutant tumour suppressor alleles are usually recessive, and loss-of-function of both the maternal and paternal allele of a tumour suppressor gene is generally required to confer a selective advantage to the cell, following the two-hit hypothesis (Knudson 1971). However, haploinsufficiency occurs when one allele is insufficient to confer the full functionality produced from two wild-type alleles (Stern 1943). The first tumour suppressor protein discovered was the retinoblastoma protein (pRb) in human retinoblastoma (Friend et al. 1986). Another important tumour suppressor is the p53 tumour suppressor protein encoded by the TP53 gene (Lane and Crawford 1979; Linzer and Levine 1979). The Rb and p53 pathways are altered in a large fraction of many types of cancers, and mutations of multiple genes in the pathway have been found to be mutated in more than one type of cancer.

A third class of cancer genes, called stability genes, are involved in DNA repair during normal DNA replication, and are responsible for keeping genetic alterations to a minimum. Unlike oncogenes and tumour suppressor genes, repair genes do not control cell proliferation or apoptosis directly, but inactivation of stability genes increase the mutation rate of other

genes. DNA repair genes include the mismatch repair (MMR), nucleotide-excision repair (NER) and base-excision repair (BER) genes (Vogelstein et al. 2004).

## Epigenetic regulation of gene expression

The term epigenetics (epi – above) describes the heritability of a phenotype, introduced by Conrad Waddington in the 1940s (Waddington 1942) . Today, an epigentic trait is defined as "a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" (Berger et al. 2009). Important components of epigenetics are DNA methylation, the modification of histone tails including acetylation and deacetylation and RNA-mediated silencing (Figure 3).

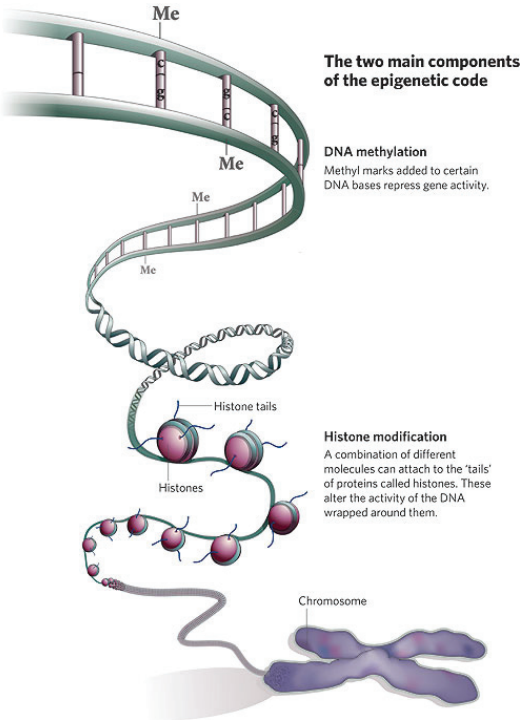


**Figure 3.** Epigenetic regulation of gene expression. Reprinted by permission from Macmillan Publishers Ltd, Nature (Qiu 2006), copyright.

### DNA methylation in cancer

DNA methylation in vertebrates is a well-established epigenetic mechanism that controls a variety of important developmental functions including X chromosome inactivation (reviewed

by (Ohhata and Wutz 2012), genomic imprinting and transcriptional regulation (discussed below).

Genomic imprinting involves DNA methylation and histone modifications in order to achieve monoallelic gene expression. The expressed allele is dependent upon its parental origin, giving rice to maternal or paternal imprinted genes in the germline which is further inherited to the offsprings. Loss of heterozygosity (LOH) at an imprinted locus may result in the deletion of the only functional copy of an imprinted tumour suppressor. Similarly loss of imprinting (LOI) at an imprinted locus may result in an increased expression of an imprinted proto-oncogene. For example, LOI of insulin-like growth factor 2 (IGF2) has been associated with an increased risk of cancer (Cui 2007). Both LOH and LOI may cause cancer (reviewed by (Jirtle 1999)) following the Knudson multiple-hit hypothesis (Knudson 1971).

DNA methylation involves a covalent modification of DNA catalyzed by DNA methyltransferases (DNMTs) and involves the transfer of a methyl group to the 5′ position of cytosine residues at 5'-CpG-3' dinucleotides (CpG). There are four types of DNMTs; DNMT1 is responsible for the maintenance of methylation (Hermann et al. 2004), DNMT3A and DNMT3B carry out *de novo* methylation (Okano et al. 1998), and DNMT3L regulates the activity of the other methyltransferases (Holz-Schietinger and Reich 2010). Gene expression may be repressed by methylation by two separate mechanisms. In direct inhibition, DNA methylation can repress transcription by impairing the binding of transcription factors to their recognition sites (Tate and Bird 1993). The second, likely most important, method is indirect inhibition, in which methylation-binding proteins (MBDs) recruits histone deacetylases (HDACs) and other chromatin remodelling proteins to the chromosome and cause chromatin compaction (Fournier et al. 2012).

CpG islands (CGIs) are clusters of CpG dinucleotides that mostly remain unmethylated under normal conditions and could play an important role in gene regulation. The decreased occurrence of CpGs in the genome is best explained by the fact that methylated cytosines are mutational hotspots resulting from the spontaneous deamination of 5-methylcytosine to thymine (Coulondre et al. 1978) leading to CpG depletion during evolution. The generally accepted definition of what constitutes a CGI was proposed in 1987 as being a DNA sequence

>200 bp with a GC content >50% and an observed:expected CpG ratio of more than 0.6 (Gardiner-Garden and Frommer 1987). A more stringent definition limiting CGIs to regions of DNA >500 bp with a GC content ≥55% and an observed:expected CpG ratio of 0.65 identified CGIs more likely to be associated with the 5' regions of genes and excluded most Alu-repetitive elements (Takai and Jones 2002). However, it has more recently been shown that most tissue- and cancer-specific DNA methylation occurs at non-promoter CGI shores, regions of up to 2 kilobases (kb) with low CG density, located next to CGIs or CpG-enriched sequences, and a strong relationship between differential methylation in CGI shores and transcription of associated genes was observed (Doi et al. 2009; Irizarry et al. 2009). CGIs encompass the transcription start site of the about half of the human protein coding genes, but approximately half of the CGIs in mammals are not associated with annotated promoters, so called "orphans", located in intergenic or intragenic regions. During development, *de novo* methylation preferentially takes place at orphan CGIs, while cancer-specific *de novo* methylation affects both orphan and promoter CGIs equally (Illingworth et al. 2010).

Alterations in DNA methylation are known to cooperate with genetic events and to be involved in human carcinogenesis. Two forms of aberrant DNA methylation are found in human cancer: hypomethylation, the overall loss of 5-methyl-cytosine, and gene promoter-associated hypermethylation. Hypermethylation of CGIs in gene promoters mediates epigenetic gene silencing in cancer, and occurs fundamentally in tumour suppressor genes. Hypomethylation of transcriptional regulatory regions in cancer has been reported to be involved in oncogene activation (Li et al. 2009), but is much less frequent than hypermethylation of CGI overlapping promoters, and is often associated with decreases in overall genomic methylation (reviewed by (Ehrlich 2009). Furthermore, mutational silencing of one allele may be followed by epigenetic silencing of the other allele resulting in inactivation of the tumour suppressor gene, as observed for the cyclin-dependent kinase inhibitor 2A (*CDKN2A*), *PTEN* and *RB1*. Deregulated epigenetic mechanisms may also generate genetic instability, causing activating mutations in oncogenes or silencing in tumour suppressors, and could be responsible for the deregulation of differentiation and proliferation programs.

### Epigenetics and clinical applications

There is growing excitement regarding the reversal of epigenetic abnormalities for cancer therapy. Both DNA demethylating and HDAC inhibition agents have shown clinical efficacy, albeit their severe side effects cannot be ignored as they both will make the whole genome hypomethylated. Low doses of azacitidine (or 5-azacytidine), which inhibit all three biologically active DNA methyltransferases (Jones and Taylor 1980), have been used for patients with myelodisplastic syndrome (MDS). Treatment gave an increase in the time of conversion of MDS to leukaemia, as well as increased overall survival (Fenaux et al. 2009). The approval of the drugs 5-azacytidine and its deoxy derivative decitabine by the US Food and Drug Administration (FDA) paves the way for refining the use of low-dose regimens not only for leukaemia, but also for solid tumours. A combination of azacitidine, marketed as Vidaza, and the HDAC inhibitor Entinostat has reach a phase II trial for advanced non–small cell lung cancer (Kaiser 2010).

## miRNAs

miRNAs are small, non-coding RNA molecules that are highly conserved across species and play key roles as regulators of gene expression. The first miRNA was identified in 1993, when Lee and Ambros discovered that the gene *lin-4* did not encode a protein, but instead produced a pair of small RNAs. These small RNAs bound to the 3' untranslated region (UTR) of the *lin-14* mRNA and caused translational repression (Lee et al. 1993). miRNAs have been estimated to regulate 30-60% of the human protein coding genes (Lewis et al. 2005; Friedman et al. 2009), and modulate the level of proteins involved in most biological processes, including cell cycle regulation, development, metabolism, apoptosis, differentiation and pluripotency (Harfe 2005; Boehm and Slack 2006; Carleton et al. 2007; Ivey and Srivastava 2010; Lima et al. 2011; Leonardo et al. 2012).

The first evidence of a correlation between miRNAs and cancer was reported by Calin et al., who observed knockdown or knockout of miR-15a and miR-16-1 in approximately 70% of all chronic lymphocytic leukemia (CLL) patients (Calin et al. 2002). Many studies have later shown that miRNAs are aberrantly regulated in human cancers, suggesting a role as a novel class of oncogenes and tumour suppressors. miRNA expression profiles can distinguish tumours from corresponding normal tissues, as well as by their developmental origin and

differentiation state (Lu et al. 2005; Volinia et al. 2006; Subramanian et al. 2007), and have shown distinct patterns in different normal tissues (Liang et al. 2007).

### Organisation of miRNA genes

Many of the known miRNAs appear in clusters through many different regions of the genome. In many cases, such closely clustered miRNAs are processed from the same polycistronic precursor transcript; a single mRNA molecule produced from the transcription of several tandem arranged genes (Lee et al. 2002). These miRNAs may show strongly correlated expression patterns (Baskerville and Bartel 2005). miRNA precursors are most frequently encoded within intergenic regions and introns of protein-coding genes, but may also be found within exons of transcripts and in antisense transcripts. Intronic miRNAs are located within a host gene and are frequently transcribed along with the primary transcript by the same promoter (Rodriguez et al. 2004). Intronic miRNAs and their host genes are shown to have correlated expression patterns (Baskerville et al. 2005). However, it has been observed that one-third of intronic miRNAs have transcription initiation regions independent from their host promoters (Ozsolak et al. 2008). Intergenic miRNA presumably rely on their own promoters (Saini et al. 2007).

The miRNAs from the miR-17 cluster; miR-17, miR-18, miR-19a, miR-19b, miR-20, miR-25, miR-92, miR-93, miR-106a, and miR-106b, is a well known example of miRNAs encoded in polycistronic transcripts. Mammals have three paralogs of the miR-17 cluster. The human miR-17-92 cluster contains six precursor miRNA within about 1 kb on chromosome 13. The second cluster (miR-106a-92) is located on the X-chromosome, and the third cluster (miR-106b-25) is a distant relative located on chromosome 7. The ancestral miR-17 cluster probably contained the ancestors of the miR-17 group, the miR-19 group, as well as the ancient miR-92. The microRNAs of the three paralog clusters arose through a complex history of tandem duplications and loss of individual members as well as duplications of entire clusters. The complex history of the miR-17 miRNA family appears to be closely linked to the early evolution of the vertebrate lineage (Reviewed in (Tanzer and Stadler 2004)).

When clustered miRNAs are of similar sequence, they may contribute additively to the regulation of a set of mRNA targets. Clusters can also contain miRNAs of distinct sequences, suggesting that the distinct miRNAs are coordinated towards their various targets. Thus, a concerted action could ensure a synergistic, redundant and more flexible means of regulation and have a higher potential to influence complex cell signalling networks. Deregulated miRNA clusters have been shown to be significantly overrepresented compared to single miRNAs in most investigated diseases (Ruepp et al. 2010).

### miRNA biogenesis

The biogenesis of miRNAs (Figure 4) starts in the nucleus, where the miRNAs are transcribed by RNA polymerase II (Lee et al. 2004) or less frequently by polymerase III in an Alu-dependent mechanism (Borchert et al. 2006). This produces long primary precursors (pri-miRNAs) up to several kilobases with monocistronic or polycistronic stem-loop RNA structures (Lee et al. 2002). Long, capped and polyadenylated pri-miRNAs (*Cai et al., 2004*) are processed by a RNAse III Drosha and by the double-stranded DNA binding protein DGCR8/Pasha into 70- to 100 nucleotides long pre-miRNAs (Ambros 2004). These precursor molecules are exported by an Exportin 5 mediated mechanism to the cytoplasm. Processing by the RNAse III Dicer generates a dsmiRNA duplex of 20-22 nucleotides, named miR/miR*, from each individual hairpin-forming miRNA transcript. The active miRNA is preferentially incorporated into the complex known as miRNA-induced silencing complex (miRISC). Argonaute (AGO) proteins, which directly interact with miRNAs, and glycine-tryptophan protein of 182 kDa (GW182) proteins, which act as downstream effectors in the repression, are key factors in the assembly and function of miRISC. According to the canonical model, the mature miRNAs of the miRISC complex are able to regulate gene expression at the post-transcriptional level through partial complementary binding to specific mRNA targets in the cytoplasm (reviewed in (Bartel 2009)). However, accumulating evidence has demonstrated an alternative non-canonical pathway where certain mature miRNAs can re-enter the nucleus to regulate the biogenesis and function of non-coding RNAs (ncRNAs), including miRNAs and long ncRNAs. (reviewed by (Chen et al. 2012)).

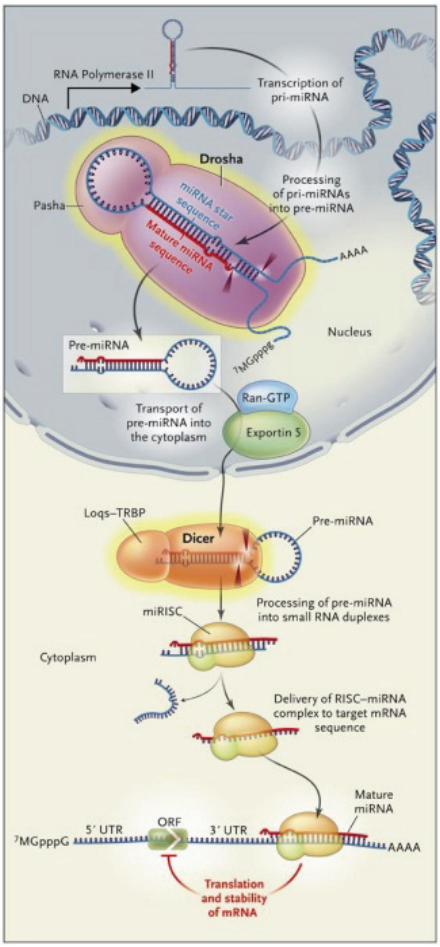


**Figure 4.** miRNA biogenesis (see text for details). Reproduced with permission from (Slack and Weidhaas 2008), Copyright Massachusetts Medical Society

One aspect of miRNA diversity comes from the ability to produce two distinct miRNAs, miR and miR*, from a given hairpin pre-miRNA. One of the miRNAs of the duplex is located 5' and the other 3' on the precursor. The standard nomenclature for miRNAs assigns the asterisk to the less abundant of the two forms found in the first identifying study. One of the strands, miR, of the hairpin loop has been thought to be involved in gene regulation, whereas the other strand, miR*, is expected to be less active and more frequently degraded. However, more recent findings have revealed that these complementary miR*s are more abundant than previously assumed (Kuchenbauer et al. 2008). It has also been shown that well conserved miR* strands are biological active (Guo and Lu 2010; Yang et al. 2010), and that the strand that makes the dominant product can change in different tissues (Ro et al. 2007). In light of

this, the miR/miR* nomenclature are gradually retired in favour of the -5p/-3p nomenclature, and available deep-sequencing data make clear which of the mature products are dominant. The database miRBase (www.mirbase.org) contains information on published miRNA sequences and annotation. In miRBase, mir represents a predicted hairpin portion of a miRNA transcript while the mature miRNA sequence is termed miR.

**Regulation by miRNAs**

The miRNAs imperfectly base-pairs mainly to the 3'-UTR of target mRNA. This binding directs gene silencing via translational repression or mRNA deadenylation and degradation. Efficient mRNA targeting requires continuous base-pairing of miRNA nucleotides 2 to 8 (the seed region), and stabilizing complementarity in the 3' UTR. Generally, miRNA-mRNA duplexes contain mismatches and bulges in the central region (miRNA positions 10–12) that prevent endonucleolytic cleavage of mRNA by an RNAi mechanism (Filipowicz et al. 2008). Multiple miRNA targets databases are available for predicting mRNA targets for specific miRNAs (Alexiou et al. 2009; Witkos et al. 2011).

The repression of many miRNA targets is frequently associated with their destabilization. Deadenylation of mRNAs is mediated by interactions between GW182 and the AGO and poly(A) binding protein (PABR), and the mRNA is degraded, which also lead to an indirect effect on protein levels (Eulalio et al. 2009; Fabian et al. 2010). The interaction between the miRISC complex and the mRNA can also have a direct effect on protein translation. miRNAs have been found to repress translation at initiation, either by targeting the cap recognition step or by inhibiting ribosome 80S complex assembly. Repression at post-initiation steps has also been reported like premature ribosome drop off, interrupted elongation, or cotranslational protein degradation. The components of miRISC (including miRNAs as well as AGO and GW182 proteins) and repressed mRNAs are enriched in processing bodies (P bodies), which are cytoplasmic structures thought to be involved in the storage or degradation of translationally repressed mRNAs (Nilsen 2007). In an alternative process, although less frequent in mammals, miRISC containing AGO2 recognizes mRNAs bearing sites nearly perfectly complementary to miRNAs causing endonucleolytically cleavage and degradation of mRNA (Fabian et al. 2010), similar to the process of RNAi (Liu et al. 2004).

Human protein-coding genes have been under selective pressure to maintain pairing to miRNAs, and many sites that match the miRNA seed, particularly those in 3' UTRs, are preferentially conserved. miRNAs with identical seed sequences may target the same mRNA, and are grouped into miRNA families. miRNAs can be grouped into conserved or non-conserved families, where the conserved families are conserved across most mammals, but usually not beyond placental mammals.

In addition to the role of miRNAs as direct regulators of target molecules, miRNAs are directly connected with the epigenetic machinery. These miRNAs, called "epi-miRNAs", can regulate expression of components of the epigenetic machinery (reviewed by (Iorio et al. 2010)). The first epi-miRNAs described was miRNAs of the miR-29 family, directly targeting *de novo* DNA methyltransferases DNMT-3A and -3B and induce global hypomethyation of tumour cells and reactivation of methylation-silenced tumour suppressor genes (Fabbri et al. 2007; Garzon et al. 2009). Among other epi-miRNAs is miR-148, previously reported to be epigenetically modulated, involved in an epigenetic regulatory loop by directly targeting of DNMT3B (Duursma et al. 2008).

### miRNAs as oncogenes and tumour suppressor genes

miRNAs have an important role in tumourigenesis, acting as oncogenes or tumour suppressor genes depending on the targets they regulate; overexpression of an oncogenic miRNA can cause excessive repression of a target tumour suppressor gene, and conversely, silencing of a tumour-suppressive miRNA can give ectopic levels of a target oncogene. Because individual miRNAs can regulate multiple targets, it should be noted that their function may differ between tissue types, depending on which of their target genes are being expressed.

One of the first oncogenic miRNAs identified was miR-155 (Metzler et al. 2004). It is located on chromosome 21 in a non-coding RNA called the B cell integration cluster (BIC) and is highly expressed in a range of different cancers like lymphoma and leukemia (Metzler et al. 2004; Calin et al. 2005), lung, breast and pancreatic cancer (Volinia et al. 2006; Greither et al. 2010). miR-21 can function as an oncogene by blocking expression of critical apoptosis-related genes like phosphatase and tensin homolog (PTEN) (Meng et al. 2007). High levels of

miR-21 has been described in a wide range of tumours such as glioblastoma, breast, lung, colon, and osteosarcoma (Chan et al. 2005; Iorio et al. 2005; Volinia et al. 2006; Ziyan et al. 2010). High levels and amplification of the miR-17-92 and miR-106b-25 clusters have also been reported for a multitude of different cancers (He et al. 2005; Volinia et al. 2006; Petrocca et al. 2008). The miR-17-92 cluster participates in the control of cell death and proliferation driven by v-myc myelocytomatosis viral oncogene homologue (avian) (MYC) through E2F1 modulation, and dramatically accelerates lymphomagenesis in mice (He et al. 2005; Mendell 2008). In addition, haploinsufficiency of the miR-17-92 culster is responsible for developmental defects in individuals with skeletal abnormalities (de Pontual et al. 2011) and during normal development (Jevnaker et al. 2011).

The tumour suppressor miRNAs miR-15a and miR-16-1 are located in a cluster at 13q14.3, a region frequently deleted in CLL (Calin et al. 2002). miR-15a and -16 induce apoptosis in leukemic cells by directly targeting the anti-apoptotic gene *BCL2 (*Cimmino et al. *2005)*. let-7 miRNAs were among the first miRNAs discovered in *Caenorhabditis elegans* (Reinhart et al. 2000), and are frequently mapped to regions within the human genome that are altered or deleted in various cancers (Calin et al. 2004), and has been shown to regulate onco-genes such high mobility group A2 (*HMGA2*) (Mayr et al. 2007) and the Harvey rat sarcoma viral oncogene homolog (*RAS*) genes (Johnson et al. 2005). The miR-29 family (comprising miR-29a, -29b and -29c), located at chromosome 7q32, is downregulated or mutated in lung cancer, CLL and osteosarcoma (Calin et al. 2005; Yanaihara et al. 2006; Jones et al. 2012). The miR-34 family (comprising miR-34a, -34b and -34c) has been identified as a component of the p53 network, and their upregulation induces apoptosis and cell-cycle arrest (Chang et al. 2007; He et al. 2007).

### Regulation of miRNAs

Control of miRNA expression and processing have emerged as important mechanisms for defining the pattern of miRNA expression (Figure 5). Just like protein coding genes, miRNA expression can be regulated by mechanisms that are commonly involved in cancers (reviewed by(Iorio et al. 2010)) like mutations, chromosomal abberations and epigenetic changes (Calin et al. 2002; Calin et al. 2005; Saito et al. 2006). miRNA genes are frequently located in

regions of loss of heterozygosity, regions of amplification, or common breakpoint regions (Calin et al. 2004). Mutations, like the inherited mutations in the primary transcripts of miR-15a and miR-16-1 in CLL (Calin et al. 2005), and single nucleotide polymorphisms (SNPs) as described in lung cancer (Hu et al. 2008), both cause deregulation of miRNAs. miRNAs are subject to the altered activity of different transcription factors like the induction of the miR-17-92 cluster by MYC (O'Donnell et al. 2005; Thayanithy et al. 2012b). In addition, also epigenetic mechanisms, such as promoter methylation or histone acetylation, can modulate miRNA expression, and an aberrant regulation at this level is found in different diseases, including cancer. The group of Peter Jones reported a strong upregulation of miR-127 in T24 bladder cancer upon treatment with 5-Aza (Saito et al., 2006). Similarly, miR-9-1 was shown to be induced in breast cancer (Lehmann et al., 2008) as well as miR-34b and miR-34c in colon cancer (Toyota et al, 2008). Conversely, upregulation of putative oncogenic microRNAs can result from DNA hypomethylation, as shown in lung adenocarcinoma for let-7a-3 (Brueckner et al, 2007) or epithelial ovarian cancer for miR-21 (Iorio et al, 2007).

The deregulated miRNA expression in cancer can also be due to defects in the miRNA biogenesis machinery (Figure 5), as supported by the changes in miRNA levels consequent to altered Drosha or Dicer activity in different tumour types (Karube et al. 2005; Thomson et al. 2006). In their role in miRNA maturation both Drosha and Dicer are assisted by a number of cofactors or accessory proteins, with some playing an important regulatory function. Likewise, the formation of the miRISC and the execution of its activity involve many additional factors. The miRNA pathway is also extensively controlled at steps downstream of miRNA biogenesis. In addition to the miRISC core components, AGO and GW182 proteins, the most obvious targets for regulation, dozens of other proteins have been identified which are implicated in positive or negative control of miRNA effects (Krol et al. 2010).

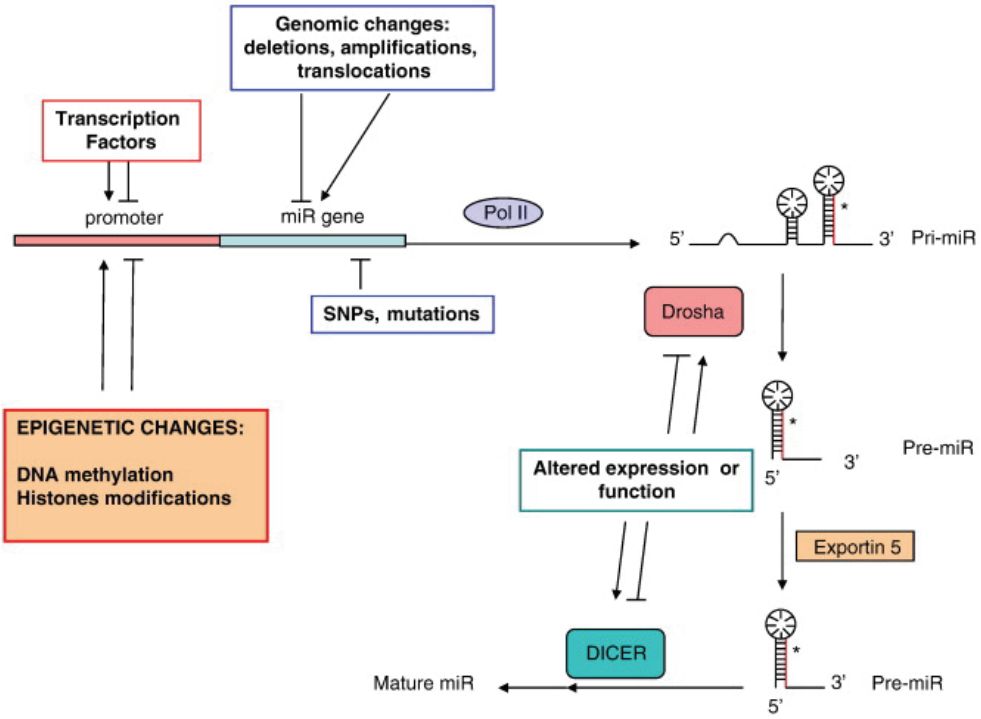


**Figure 5.** Regulation of miRNA expression. Reproduced with permission from (Iorio et al. 2010), Copyright Elsevier.

## miRNAs in anti-cancer therapy

miRNAs are attractive targets for development of anti-cancer gene therapy (reviewed by (Iorio and Croce 2012), either alone or in combination with current targeted therapies. Re-expression of miRNAs downregulated in cancer or silencing of upregulated miRNAs may lead to cancer cell apoptosis and exert a therapeutic effect. One of the most appealing property of miRNAs as therapeutic agents is their ability to target multiple molecules in cancer-related pathways (O'Day and Lal 2010). After the initial discovery and validation of miRNAs playing crucial roles in a specific cancer type, the main issues for a success in anti-cancer therapy are development of specific methods for drug delivery and avoidance of unwanted off target effects. A recent study demonstrated that a locked nucleic acid (LNA)-modified oligonucleotide (SPC3649) complementary to miR-122 dramatically reduced hepatitis C virus in the liver and in the bloodstream in chronically infected chimpanzees (Lanford et al. 2010). As the first miRNA-targeted drug to enter human clinical trials, SPC3649 (named miravirsen), was tested in phase I and II studies, and a decrease in viral

levels was observed with no significant adverse events (Janssen et al. 2011; Reesink et al. 2012). Presumably, the approach of using anti-miRNA therapeutics alone or in the combination with current therapies may soon find its way into the clinic.

## Sarcomas

Sarcomas (from the Greek sarx meaning "flesh" + oma meaning tumour) are malignant tumours arising from mesenchymal progenitor cells. Sarcomas are a heterogeneous group of cancers, given their names based on the type of tissue which they resemble. More than 50 different types of sarcoma have been described, and sarcomas are divided into the two main categories; soft-tissue sarcoma and bone sarcomas. Soft-tissue sarcomas most commonly develop from and within the connective tissues like muscle, adipose, neural, vascular, fibrous and lymphatic tissues. The most common soft-tissue sarcomas are liposarcomas, leiomyosarcoma, synovial sarcomas and malignant peripheral nerve sheath tumours. Bone sarcomas; primary malignant tumours originating in bone, represent about 0.2% of all cancer cases world-wide (Dorfman and Czerniak 1995). The most common bone sarcomas are; chondrosarcoma, osteosarcoma and Ewing' sarcoma – with falling incidences in that order. Bone sarcomas are more common in children and young adolescents. According to the Norwegian Cancer Registry, there were 62 new cases of bone sarcomas in Norway in 2009, and they accounted for about 6% of cancers within the age-group 5-19 years.

Sarcomas can be divided into two main groups depending on their chromosomal aberrations. One group consists of tumours with small and subtle alterations, showing a specific translocation, a few gain and losses or specific gene mutations (*e.g.* Ewing sarcoma, synovial sarcoma, myxoid liposarcoma and gastrointestinal stromal tumours). The other group consists of tumours with extensive alterations, showing complex karyotypes with numerous gains and losses without specific genetic alterations (*e.g.* osteosarcoma, chondrosarcoma and leiomyosarcoma) (Bovee and Hogendoorn 2009).

### Osteosarcoma in general

Osteosarcoma is the most common bone cancer among children and adolescents. Males are more frequently affected than females in a ratio of 3:2 (Aksnes et al. 2006). The peak age of

incidence coincides with a period related to growth spurt in young people, a feature that suggest a relationship between rapid bone growth and the development of osteosarcoma. A secondary smaller peak incidence is seen in patients over 65 years of age. However for these late onset osteosarcomas predisposing factors like previous radiatioterapy, genetic condition (Li-Fraumeni syndrome, hereditary retinoblastoma; RB), bone diseases (*e.g.* Paget disease of bone) can often be causal. Osteosarcomas usually arise in the long bones of the extremities near the metaphyseal regions. The most common sites are the femur (42%), tibia (19%) and the humerus (10%), skull or jaw (8%) and the pelvis (8%) (Ottaviani and Jaffe 2009) (Figure 6). Tumours with various patterns of differentiation are traditionally referred to as histological subtypes. Conventional osteosarcoma is a high grade malignant primary central tumour characterized by the presence of osteoid extracellular matrix, and represents approximately 75% of all osteosarcomas including the chondroblastic, fibroblastic and osteoblastic histological subtypes. This classification has, however, little impact on clinical decision making because there is no statistical difference in the survival outcomes of patients with high-grade tumours of these three histological types, and the treatment for all types is the same (Klein and Siegal 2006). According to the World Health Organization (WHO) classification, the rarer subtypes include teleangietatic, small cell, parosteal, periosteal, low-grade central, secondary and high-grade surface osteosarcoma (Fletcher 2002). Osteosarcoma may also develop at an extraskeletal site, accounting for fewer than 2% of all soft tissue sarcomas (Rosenberg and Heim 2002).

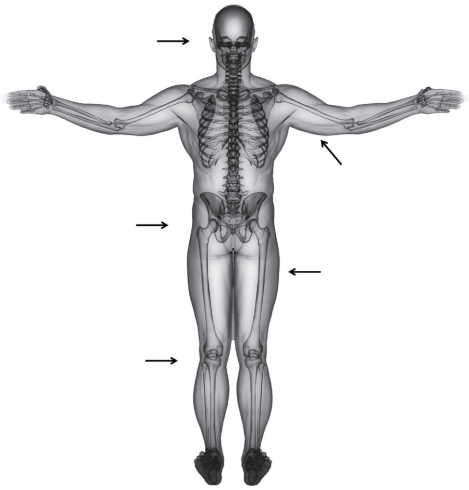

**Figure 6.** Predilections of osteosarcoma. The most common sites for osteosarcoma are the femur (42%), tibia (19%) and the humerus (10%), skull or jaw (8%) and the pelvis (8%). Photo Shutterstock (www.shutterstock.com).

Conventional osteosarcomas are high-grade tumours; most with a complex karyotype that displays numerous genetic aberrations (Raymond et al. 2002). Currently used chemotherapy (*e.g.* Methotrexate, Cisplatin and Doxorubicin usually administered both pre- and postoperatively) has significantly improved survival. However, in selected subgroups the prognosis is still dismal and chemotherapy is often associated with severe toxicity (Bruland and Pihl 1997; Bruland et al. 2009a). There have been advances in multimodal treatment combining surgical-wide resection with limb-sparing surgery in more than 90% of the patients, adjuvant chemotherapy or radiotherapy in combination. However, the 5-year metastasis-free survival rate for patients diagnosed with osteosarcoma without presence of metastasis remains in the order of 60-70%, and sarcoma-related survival of 70-75% (Smeland et al. 2003; Ferrari et al. 2005; Smeland et al. 2011). The 5-year survival rate for osteosarcoma patients with primary metastases or metastatic relapse is in the range of 10-28% (Bacci et al. 2003; Kager et al. 2003; PosthumaDeBoer et al. 2011). Due to the unacceptable adverse effects and frequent failure of prevailing therapy there is a need for new therapeutic modalities in sarcoma.

### Metastasis in osteosarcoma

Metastases are the leading cause of cancer-related death. Around 13-27% of the osteosarcoma patients have detectable metastases at diagnosis (Bielack et al. 2002; Aksnes et al. 2006; Hattinger et al. 2010; PosthumaDeBoer et al. 2011), whereas 40% will develop metastases at a later stage (PosthumaDeBoer et al. 2011), most often as lung metastases. In the last 20 years, modifications to the standard treatments for these patients have not made any significant changes in prognosis. Molecular alterations present in metastatic cells may contribute to resistance to chemotherapy, and targeting these features might enhance the efficacy of current treatments. In order to develop targeted therapies that can improve outcomes for the patients, it is important to identify the key genes or proteins that promote metastasis in osteosarcoma. The molecular alterations contributing to metastasis in osteosarcomas are increasingly being understood. An extensive overview of the biology of metastatic osteosarcoma cells and the preclinical and clinical efforts targeting the different steps in the metastatic process in osteosarcoma have recently been presented (PosthumaDeBoer et al. 2011).

The metastatic process in osteosarcoma is haematogenous (Bruland et al. 2005; Bruland et al. 2009b) and shows a tropism for lungs (80%), with skeleton as the second most common site (PosthumaDeBoer et al. 2011). It is suggested that the circulating cancer cells direct towards a specific metastatic site and interact with the surface molecules on the endothelium at the secondary organ. Chemokines are chemotactic cytokines, a family of small cytokines or proteins secreted by cells. The major role of chemokines is to control and direct the migration of cells, and it has been increasingly known that chemokines play an important part in the metastatic cascade in a wide range of cancers, including osteosarcomas (Muller et al. 2001; Laverdiere et al. 2005; Bennani-Baiti et al. 2010; Singh et al. 2011). Cancer cells that are attracted by chemokines have an increased expression of particular G-protein coupled receptors on their surface. The cells migrate towards a signal of increasing chemokine concentration provided by the source of the chemokine, and this process enables them to migrate to secondary tissues where chemokine ligands are highly expressed (Figure 7). The chemokine (C-X-C motif) receptor 4 (CXCR4) is a specific receptor for the ligand chemokine (C-X-C motif) ligand 12 (CXCL12), and the CXCR4/CXCL12 axis has been shown to be important for progression in a high number of cancer types (Balkwill 2004). In a mouse model, the cancer cells with CXCR4 receptor were chemoattracted by CXCL12, migrated through the lymphatic and vascular system, and arrested in CXCL12 rich organs like the bone and lungs (Perissinotto et al. 2005).

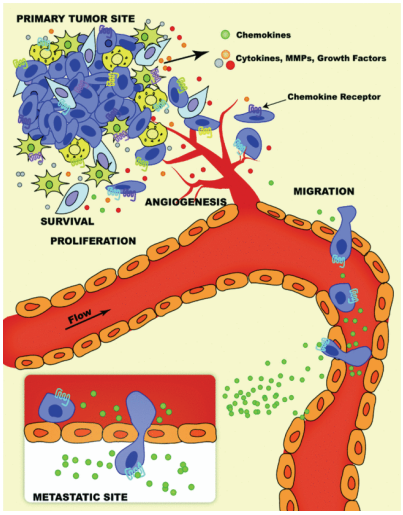


**Figure 7.** Illustration of the various steps in cancer growth and metastasis where chemokines and receptors play a role. Reproduced with permission from (O'Hayre et al. 2008), copyright Biochemical Society.

## Genetic changes in osteosarcoma

At the molecular level osteosarcomas show complex genomic aberrations and highly variable patterns of gene expression between individual tumours, which make it difficult to identify the molecular features that underlie the development of this type of cancer.

### *Genomic aberrations and mutations*

Osteosaroma is characterized by a high level of chromosomal instability leading to copy number alterations, such as polyploidy, aneuploidy, amplifications, deletions and unbalanced translocations. A vast number of DNA copy number changes have been identified using chromosome comparative genomic hybridization (CGH), higher resolution microarray-based array CGH, and more recently also utilizing high-density SNP microarrays (Yen et al. 2009; Kresse et al. 2010; Smida et al. 2010). Few consistent chromosomal aberrations have been recognized in osteosarcoma, mainly consisting of recurrent alterations in 6p, 8q, 13q and 17p (Man et al. 2004; Kresse et al. 2009; Yen et al. 2009; Smida et al. 2010).

The tumour suppressor pathways p53 and RB are frequently targets for inactivation in osteosarcoma. Both pathways comprise many upstream regulators and downstream effectors and interact and cross-regulate with each other at several points. The RB pathway directly regulates the cell cycle and hence cellular senescence, but is also important in apoptosis, probably by interacting with the p53 pathway. The tumour suppressor pRb, (encoded by the *RB1* gene at 13q14), is the central player in the RB pathway, and *RB1* is frequently deleted or mutated in osteosarcoma (Wadayama et al. 1994; Kresse et al. 2009). The *CDKN2A* gene at 9p21 encodes the proteins $p16^{INK4a}$ and $p14^{ARF}$. $p16^{INK4a}$ inhibits the phosphorylation of pRb through regulation of cyclin-dependent kinase 4 (CDK4) and 6 (CDK6) (Stott et al. 1998). The tumour suppressor *CDKN2A* has been shown to be deleted with loss of p16 expression in osteosarcomas that have no *RB1* alterations (Nielsen et al. 1998). A significant association has been found between CDKN2A/p16 expression and overall survival of patients (Mohseny et al. 2009).

The p53 protein, encoded by the transcription factor p53 gene (*TP53*, 17p13.1), is a well known tumour suppressor, and plays an essential role in the regulation of apoptosis and

cellular senescence. In response to stress, such as DNA damage and oncogene activation, p53 is post-translational stabilized and activated as a transcription factor to turn on or off the expression of different sets of downstream target genes that facilitate survival or death of damaged cells. p53 is non-functional in a majority of cancers, and is directly inactivated by mutations in about 50% of the cases. In addition, tumours retaining wild-type p53 (*TP53*$^{Wt}$) might have defects in other parts of the p53 network, resulting in disabled p53 function (Vogelstein et al. 2000). *TP53* mutation is detected in approximately 20% of high-grade central osteosarcomas, but has not been associated with clinical outcome (Wunder et al. 2005), contrary to the observation in many other malignancies. However, the mutation of *TP53* correlates significantly with the presence of high levels of genomic instability in osteosarcomas (Overholtzer et al. 2003).

In *TP53*$^{Wt}$ tumours, p53 may be inactivated by the mouse double minute 2 (MDM2, 12q15) oncogene. Amplification of the MDM2 amplicon occurs in about 10% of high-grade, classical osteosarcomas (Mejia-Guerrero et al. 2010). Overexpressed MDM2 protein controls p53 function by inhibiting p53-mediated transcriptional activity (Momand et al. 1992) and by acting as an ubiquitin ligase promoting p53 degradation (Haupt et al. 1997). The p14ARF protein binds to the p53/MDM2 complex and inhibits MDM2-mediated degradation of p53 (Stott et al. 1998). Restoring p53 activity by inhibiting the interaction between p53 and MDM2 offers an attractive approach to cancer therapy. The small-molecule inhibitor Nutlin-3a has been shown to suppress proliferation and promote apoptosis through activation of the p53 pathway in osteosarcoma with *TP53*$^{Wt}$ (Wang et al. 2012a).

High-level amplification of 8q23-q24, harboring the well-known oncogene *MYC*, is frequently seen in osteosarcoma (Squire et al. 2003; Kresse et al. 2009). The multifunctional MYC protein act downstream of many signal transduction pathways, functioning as a central hub that integrates multiple intracellular and extracellular signals, and plays an important role in cell cycle progression, apoptosis and cellular transformation (reviewed by (Dang 2012)). Recurrent gain and increased RNA and protein levels of the transcription factor RUNX2 (6p21) have been seen in osteosarcoma (reviewed by (Martin et al. 2010). RUNX2 is genetically essential for developing bone and osteoblast maturation, and may be a causative factor in osteosarcoma pathogenesis. A more recent candidate in osteosarcoma, the gene

limbic system-associated membrane protein (*LSAMP*) has been reported to be a tumour suppressor. Loss of the *LSAMP* locus in 3q13.31 and low expression of *LSAMP* were significantly associated with poor survival (Kresse et al. 2009).

*Deregulated miRNAs in osteosarcoma*

miRNAs deregulated in human osteosarcoma compared to bone, osteoblasts and mesenchymal stem cells were recently (Widschwendter et al. 2004; Lulla et al. 2011; Maire et al. 2011; Baumhoer et al. 2012b; Hu et al. 2012; Thayanithy et al. 2012b) published. Among the 34 signature miRNAs described by Jones et al., miRNAs from the miR-181 family were overexpressed in osteosarcoma clinical samples compared to bone, while the expression of miR-16, miR-29b, miR-142-5, miR-223 and miR-451 was reduced where miR-16 was confirmed to act as a tumour suppressor. Thayanithy et al. reported 36 miRNAs as deregulated compared to bone where the miRNAs from the 14q32 locus showed a consistent downregulation while the miRNAs of the miR-17-92 cluster was upregulated.

Individual miRNAs of the miR-17-92, miR-106a-92 and miR-106b-25 clusters were also reported upregulated in osteosarcomas compared to osteoblast cell lines (Baumhoer et al. 2012b). Maire et al. (Maire et al. 2011) and Lulla et al (Lulla *et al*, 2011) reported 38 and 22 miRNAs, respectively, to be deregulated in human osteosarcoma clinical samples compared to osteoblasts, of which miR-126, miR-142-3p , miR-148a and miR-451 were upregulated in both studies. In addition, Lulla et al. identified miR-135b, miR-150, miR-542-5p and miR-635 to be highly expressed in osteosarcoma, while Maire et al found miRNAs at the 14q32 locus downregulated in osteosarcomas compared to osteoblasts, in line with the observations done by Thayanithy et al. in the comparison against bone. One single osteosarcoma cell line, MG-63, was compared against the hFOB1.19 osteoblast cell line, revealing overexpression of miR-9, miR-99, miR-195, miR-148a and miR-181a and downregulation of miR-143, miR-145, miR-335 and miR-539 (Hu et al. 2012). The deregulation of miR-148a was also identified by Maire et al. and Lulla et al, and miR-335 was identified by Maire et al.

Previous studies have reported deregulation of specific miRNAs in osteosarcomas (reviewed in (Kobayashi et al. 2012)). miR-140 and miR-215 have been associated with

chemoresistance (Song et al. 2009; Song et al. 2010). miR-143, down-regulated in osteosarcoma cell lines and primary tumour samples, promotes apoptosis, suppresses tumourigenicity (Zhang et al. 2010) and regulates metastasis (Osaki et al. 2011). miR-21 has been reported as overexpressed in osteosarcoma tissue where it is involved in cell invasion and migration (Ziyan et al. 2010). miR-192, miR-194 and miR-215 (Braun et al. 2008) and miRNAs of the miR-34 family (He et al. 2009) are all involved in p53-related cell cycle regulation. The publically available database S-MED contains miRNA expression data from a total of 25 different tissue sarcoma subtypes and normal samples, including osteosarcomas (Sarver et al. 2010).

## SUMMARY OF THE PAPERS

**Paper I.** *"Global gene expression profiling of human osteosarcomas reveals metastasis-associated chemokine pattern"*

Metastases are the leading cause of cancer-related death in osteosarcoma, and in cancer in general. In osteosarcoma, around 13-27% of the patients have detectable metastases at diagnosis, whereas 40% will develop metastases at a later stage, most often as lung metastases. In paper I, the differences in gene expression pattern between primary and metastatic osteosarcoma were investigated. At a global level, genes belonging to pathways involved in immunological processes and chemokine and cytokine signalling were higher expressed in metastases compared to primary samples, indicating an important role of these processes in the metastatic cascade. A comparison was done between primary samples from patients that had or had not developed metastases; primary samples from patients with a metastatic disease showed higher levels of *CXCR4* expression than those without metastases, comparable to the expression level in metastatic lesions. These findings revealed that a metastatic signature was present in the primary cells or possibly in the surrounding stroma cells, and that expression of the chemokine receptor *CXCR4* was associated with subsequent development of metastases in osteosarcoma. Limited sample number severely limits the power of the study, but to our knowledge it represents the first study where an association between *CXCR4* and metastasis has been identified using microarray experiments.

**Paper II.** *"Modulation of the Osteosarcoma Expression Phenotype by MicroRNAs"*

miRNAs are small, non-coding RNA molecules that have an central role as oncogenes or tumour suppressor genes in cancers. In order to identify miRNAs that may be important in osteosarcoma, comparisons of miRNA expression levels in osteosarcoma and normal samples were accomplished. The origin and developmental processes leading to osteosarcoma are not fully known. Osteoblasts and bone represent the pre-bone cells and the fully endpoint of bone differentiation, and were used as normal samples in our study. An analysis was performed on miRNA and mRNA microarray data, and 177 miRNAs were identified to be differentially expressed in osteosarcoma cell lines relative to normal bone. All miRNAs in the miR-17-92,

miR-106b-25 and miR-106a-92 clusters were found to be overexpressed. The most interesting inversely correlated miRNA/mRNA pairs in osteosarcoma cell lines included miR-9 and the transforming growth factor, beta receptor II (*TGFBR2*) as well as and miR-29 and the phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1/p85a). The expression level of a set of 15 miRNAs, representing a subset of the most differently expressed miRNAs identified in our study was validated in osteosarcoma patient samples, bone and osteoblasts by the use of qRT-PCR. The miRNAs miR-1, miR-9, miR-18a, miR-18b, miR-29b, miR-31, miR-126, miR-133b, miR-142-3p, miR-144, miR-195, miR-223, miR-451 and miR-497 were significantly changed in osteosarcoma compared to bone and/or osteoblasts. Interestingly, all but two of the 15 miRNAs showed an intermediate expression level in osteosarcoma patient samples compared to the mean values in bone and osteoblasts. The observed differences between osteosarcoma, bone and osteoblasts in our study may both be related to the differentiation level and to the malignant process of osteosarcoma development, however all of the miRNAs that were confirmed downregulated in osteosarcoma compared to bone are known to act as tumour suppressors in other cancers.

**Paper III.** *"Epigenetic regulation of miRNA in osteosarcoma; an integrated analysis of genome-wide miRNA and DNA methylation changes"*

Hypermethylation of CpGs in gene promoters located upstream of a miRNA tumour suppressor may mediate silencing of the miRNA and concomitant upregulation of oncogenes that under normal circumstances would have been suppressed by the miRNA. In order to identify epigenetically regulated miRNA loci in osteosarcoma, genome-wide methylation and miRNA expression data for osteosarcoma cell lines, normal bone and osteoblasts were compared. Among the identified candidate miRNAs, the miRNAs miR-34b-5p, miR-34c-5p, miR-126-3p, miR-224-5p, miR-335-5p, miR-452-5p, miR-486-5p, miR-598 and miR-631 were selected for further analysis. The associated CpGs of these miRNAs were differently methylated in tumour compared to normal samples and/or showed a strong inverse correlation between miRNA and methylation levels in the samples. The miRNAs *mir-34b/c*, *mir-335* and *mir-486* were induced upon 5-Aza-2'-deoxycytidine treatment, and affected proliferation of the osteosarcoma cell line MG-63. The qMSP data showed that *mir-486* was cancer–specific methylated in osteosarcoma. An upstream CpG island of *mir-335* was confirmed to be hypermethylated in osteosarcoma patient samples compared to normal bone samples. miR-

335 has previously been shown to be involved in both osteogenic differentiation and cell proliferation. miR-486-5p and miR-335-5p represents new tumour suppressors in osteosarcoma.

# DISCUSSION

## Experimental considerations

### Biological material

*Clinical tumour samples*

The clinical tumour samples included in these studies originate from a panel of human sarcomas collected at the Norwegian Radium Hospital during the period of 1987 to 2009. The Norwegian Radium Hospital is the National competence centre for treatment of sarcoma. The information given to the patients, the written consent used, the collection of samples and the research project were approved by the Regional Ethical Committee for Southern Norway. The osteosarcoma clinical samples have been named by the anonymous letter code OS and numbered consecutively. Pathological revision was done by a sarcoma pathologist at the time of collection, and further pathological revision was performed for all tumour samples included in the papers. The samples were classified according to the current WHO classification in consensus with the Scandinavian Sarcoma Group (SSG). Anonymous, updated clinical information of the patients was obtained from the MEDinsight database at the Norwegian Radium Hospital.

The clinical samples were obtained from untreated open biopsies used for diagnosis or ffrom surgical specimens. Osteosarcoma patients are generally treated with chemotherapy before surgery. The only exception is the EuroBoNeT cohort used to generate mRNA data in paper II, where only pre-treatment biopsies of high-grade osteosarcoma patients were included. The clinicopathological details for this cohort can be found in (Kuijjer et al. 2011) and will not be discussed in this thesis. The treatment regimens have changed during the period of sample collection, and the chemotherapy has been given according to the current SSG protocols, the Italian Sarcoma Group/Scandinavian Sarcoma Group (ISG/SSG) protocol or European and American Osteosarcoma Study Group (EURAMOS) protocol (http://www.ssg-org.net/index.htm). The differences in treatment can contribute to post-treatment sample heterogeneity. Due to the limited availability of untreated samples, both treated and untreated samples were included in all three papers, but the expression pattern of the treated samples

was not significantly different from the untreated samples for the findings reported in the papers. For paper I, both primary tumours and metastases were included where the primary and metastatic samples were taken from different patients. For paper II and III the clinical samples consisted of primary tumours only, and no recurrences or metastases were included.

Patient material was carefully dissected, eliminating any traces of macroscopic normal tissue. However, the tumour is infiltrated by normal (stroma) cells, which will influence the analysis. The tumour samples may show different levels of inter tumour variability like variable degrees of necrosis, vascular invasion, and cellular pleomorphism, which may also influence the detection of genetic (Francis et al. 2005) and epigenetic alterations in the cells.

Due to the limited availability of clinical samples, the patient cohorts used in the papers are not homogenous. The majority of the included patient samples originate from the long bones (femur, fibula, tibia, humerus), but samples with a primary location in costa and pelvis scapula are also present. For paper II and III, only classical osteosarcoma tumours were included. For paper I, one sample subtyped as parosteal (OS11) and one sample subtyped as spindle cell/pleomorphic osteosarcoma (OS47) were included. OS11 expressed *CXCR4* at the same level as the other metastatic samples and did not show any deviating expression pattern. OS47 showed some differences in expression pattern compared to the remaining primary samples in paper I. OS47 was initially included in paper II, but the initial analyses showed a divergent miRNA expression profile of miR-142-3p, miR-451 and miR-223, thus this sample was removed from the tumour panel in paper II. For paper I, adolescents and adults were included with a range of 11-50 years, median age 18 (male:female, 1.3:1). For paper II, the cohort used for the miRNA experiments was more homogenous consisting of only younger patients from 12-33 years, median age 19 (male:female, 2:1). For the quantitative methylation-specific polymerase chain reaction (qMSP) experiments in paper III, the Norwegian cohort included patients from 11-64 years, median age 25 (male:female, 2.8:1), while for the methylation arrays patients in the range of 12-64 years, median 20 (male:female, 4:1), were included. More stringent inclusion criterias for the patient samples could have been applied, but this would have limited the already scarce osteosarcoma panel and reduced the power of the statistical analyses.

*In vitro and in vivo model systems*

Human cancer cell lines and xenografts are widely used as model systems in cancer research, and are important experimental tools in understanding the behaviour of the primary tumours. Some of the tumour samples have been established as xenografts; the samples have been grown subcutaneously in immunodeficient mice (Kresse et al. 2012a). The xenograft samples are denoted with an "x". Additional samples have been established as immortalized cancer cell lines that will proliferate indefinitely in contrast to normal cells that reach senescence. The use of cancer cell lines in research has several advantages, e.g. they are easy to culture and manipulate *in vitro*, yield substantial amounts of high-quality RNA and DNA. An additional advantage is the commercial availability of a broad series of tumour types. Although primary tumours are heterogeneous and are, to different degrees, contaminated with normal cells, tumour cell lines are usually free of normal cells. During the *in vivo* growth in mice or in *in vitro* cell systems, the tumour cells interact with a different microenvironment and the cells are subject to a selection pressure to maintain proliferation and survival, and are thus more likely to select for a more aggressive phenotype.

Whether observed gene expression and methylation patterns are a consequence of the manipulation of cell lines or xenografts or whether they represent cancer-specific characteristics remains a matter of debate. The osteosarcoma cell line and xenografts used in this thesis have been thoroughly characterized at the genomic level, and genetic and phenotypic characterization have shown that these cell lines and xenografts robustly represent osteosarcoma clinical samples (Mayordomo et al. 2010; Ottaviano et al. 2010; Kuijjer et al. 2011; Mohseny et al. 2011b; Kresse et al. 2012a; Kresse et al. 2012b).

*Normal samples*

Although it is often anticipated that osteosarcoma develops from immature bone cells, the origin and developmental processes leading to osteosarcoma are not fully known. Osteoblasts and bone represent the pre-bone cells and the fully endpoint of bone differentiation, respectively. Hence, we have included primary osteoblasts and normal bone in our study. While osteoblasts represent *in vitro* cultures, the bones are *in vivo* samples.

Normal samples were not easily available for the study, and the small number of normal samples introduces some uncertainty into the data. In total, seven bone samples have been included in the three papers. Bone1, Bone2, Bone5, and Bone7 were obtained from amputations of cancer patients at the Norwegian Radium Hospital, and Bone6 from University College London, and the normal samples were collected distant from the margin of the tumour. The bone samples originated from the long bones (femur, tibia, fibula) and the clavicle. The age of the patients was in the range of 4-72 years, two females and 3 males. Two bones were purchased from Capital Biosciences (Maryland, USA); Bone3 from a 26 year old male and Bone4 from a 76 year old female. Bone 1-4 showed normal diploid karyotype (Kresse et al. 2012b).

Commercially available primary osteoblast cultures were included, isolated from human calvaria (OB1 and OB2) (Sciencell Research Laboratories, California, USA) and from femur and tibia of different donors (OB3, OB4, OB6, OB7, OB8) (Cambrex BioScience, Maryland, USA). The osteoblasts originating from calvaria or long bones showed similar patterns of gene expression for the findings reported in paper II and III.

### Microarray experiments

Microarrays are a standard laboratory technique for high-throughput gene expression profiling in genomics research. In paper I, global mRNA microarray experiments were performed using the Human Genome Survey Microarray V2.0 slides from Applied Biosystems containing 32,878 probes for the interrogation of 29,098 genes. Whenever possible, probes are designed against common regions of multiple alternative transcripts for a particular gene. In some cases, to ensure that all the transcripts for a particular gene can be assayed, multiple probes were designed to that gene. Eighty-seven percent of the genes on the microarray are targeted by a single probe. Most of the probes, which are 60-mers, are found within 1,500 bases of the 3' end of the source transcript, where labelling is more robust. One sample is assayed per slide.

In paper II, global mRNA microarray experiments were performed using the Illumina Human-6 v2.0 Expression BeadChip with around 48,000 transcript probes per sample. The BeadChip

arrays are arranged in a multi-sample format allowing six samples to be processed in parallel, for higher throughput and reduced sample-to sample variability. Each BeadArray consists of randomly packed beads, each bead bearing many copies of a particular 50-mer oligonucleotide sequence (the "probe") to ensure that robust measures of expression can be obtained. The probes are designed to interrogate the majority of protein-coding transcripts in humans along with a large set of both positive and negative control probes.

In paper II and III, global miRNA microarray experiments were performed using the Agilent Human miRNA Microarrays v2 covering 799 human miRNAs. 40–60-mer oligonucleotide probes are *in situ*-synthesized directly on the array. The small size and high sequence homology of miRNAs represents a unique challenge for hybridization-based detection methods. Inclusion of an additional G-C pair in the probe-target interaction region stabilizes targeted miRNAs relative to homologous RNAs. In addition, all probes contain a 5' hairpin, abutting the probe-target region, to increase target and size miRNA specificity and sensitivity. Some initial publications showed that cross hybridization could occur between miRNAs with high sequence homology (Wang et al. 2007), but introduction of empirical Tm balancing significantly improved the specificity of the array probes (Agilent Technical Support, personal communication). The use of methylation arrays in paper III is discussed further back in the section describing validation of methylation.

Quality control of sample RNA and data is an important step when performing any microarray gene expression study. RNA purity was measured on a NanoDrop-1000 spectrophotometer (Nanodrop Technologies), and RNA integrity was evaluated on an Agilent 2100 Bioanalyzer, ensuring good quality RNA for downstream analysis. The quality control report generated from the Agilent Feature Extraction software was used to evaluate the reproducibility and reliability of the miRNA microarray data. Any individual experiment that did not fulfil the quality criteria or was identified as an outlier in a principal component analysis (PCA) plot was re-hybridized. The Illumina mRNA data were processed in Beadstudio, and arrays that did not fulfil the quality criteria or clustered as outliers were removed from the dataset.

Normalization is a pre-processing step with the purpose of removing systematic artefacts that do not represent true biological variation between samples. The choice of normalization method is a crucial step in the analysis. For all three papers, the mRNA and miRNA microarray data were normalized using quantile normalization (Bolstad et al. 2003). Quantile normalization is a widely used method and is based on the assumption that the majority of the genes is not influenced by the experiment and therefore shows an unchanged expression in the sample. Put simple, quantile normalization adjusts the overall expression levels to make the distribution for all samples equal. Quantile normalization has shown to be among the most robust procedures for normalization of Agilent miRNAs (Pradervand et al. 2009). Log2 transformation (paper I and miRNA data in paper II and III) or variance-stabilizing transformation (vst) (mRNA data in paper II) was applied on the microarray data, which has the advantage of producing a continuous spectrum of values and treating up- and downregulated genes in a similar fashion. Vst transformation (Lin et al. 2008) takes advantage of the technical replicates available on an Illumina microarray, and vst in combination with quantile normalization has been recommended for the pre-processing of Illumina BeadArrays (Dunning et al. 2008).

The microarray data were filtered to avoid the inclusion of genes that were low expressed across all subgroups of data. Small changes in low expressed genes may result in overestimated differences in expression levels albeit these genes are not biological interesting. In paper I, weakly expressed probes were filtered away by defining that a probe is only detected if it has a signal-to-noise ratio (SNR) $\geq 3$ in at least 50% of the samples in either subgroup primary or metastatic. In paper II, only miRNAs with detectable expression in at least 75% of the bone samples and/or 25% of the osteosarcoma cell lines were retained for further analysis, enabling the identification of miRNAs present in only a subgroup of the cell lines, as well as miRNAs present in the majority of bone or cell lines.

Regardless of the experiment performed, one outcome that is invariably of interest is the identification of genes that are differentially expressed in a subset of the experimental samples. In paper I, the Statistical Analysis of Microarrays (SAM) (Tusher et al. 2001) was used to identify differentially expressed genes between the primary and the metastatic samples, as well as between the primary samples that did and did not develop into metastases. SAM identifies statistically significant genes by carrying out gene specific t-tests and

computes a statistic $d_j$ for each gene $j$, which measures the strength of the relationship between gene expression and a response variable, e.g. primary or metastasis. For genes with scores greater than an adjustable threshold, SAM uses permutations of the repeated measurements to estimate the percentage of genes identified by chance, the false discovery rate (FDR). This analysis uses non-parametric statistics, since the data may not follow a normal distribution. This is an advantage over techniques which assume equal variance and/or independence of genes. In paper II, statistical tests were performed to identify miRNAs and mRNAs that were significantly differentially expressed between osteosarcoma cell lines or clinical samples and normal bone, and the p-value was Benjamini and Hochberg FDR adjusted.

### Quantiative real-time reverse transcription PCR

Quantitative real-time reverse-transcription PCR (qRT-PCR) was used to determine the expression level of *CXCR4* in paper I and the expression level of mRNAs and miRNAs in paper II and III. These genes were initially identified as differently expressed in cell lines or clinical samples by the use of microarrays. qRT-PCR was performed to validate the results using another methodology (all papers), to confirm the expression pattern in clinical samples (paper II) and to quantify the effect of 5-Aza treatment (paper III).

Reverse transcription was performed using High Capacity RNA-to-cDNA Master Mix for mRNA and The TaqMan MicroRNA Reverse Transcription Kit for miRNA quantification. cDNA synthesis is a critical step since all transcripts should be equally reverse-transcribed in order to preserve the relative amounts of the different transcripts. For mRNA gene expression, all mRNAs in a sample are simultaneously reverse transcribed. For miRNA gene expression, all miRNAs can be simultaneously transcribed by the use of a Megaplex Primer Pool. In paper II, the Megaplex Primer Pool A was used for all miRNAs, except miR-144 and miR-497 which was not included in the pool and had to be run as individual assays. Individual reverse transcription was used for all miRNAs in paper III. To ensure unbiased expression detection independent of the method used, an initial experiment was performed on both Megaplex primer pool and individual assays. The endogenous control RNU44 showed a high correlation (r=0.94) between the experiments based on an individual assay or pool.

qRT-PCR was performed using TaqMan Gene Expression and MicroRNA Assays (Applied Biosystems). For the mRNA expression assays, probes spanning an exon junction were used, avoiding amplification of genomic DNA. The PCR reaction efficiency of the assay probes may vary, and to account for this, serial dilutions of the Universal Human Reference RNA (Stratagene) was used for making a standard curve in paper I. However, no variation was observed for the reaction efficiencies for the different probes. In paper II and III, no standard curves were made for the mRNAs or miRNAs. Instead, the comparative Ct method was used to determine relative expression levels, assuming a similar reaction efficiency of all probes. In paper III, serial dilutions of *in vitro* methylated DNA were used to generate a standard curve for the qMSP calculations.

Normalization of the data is one of the main issues with qRT-PCR. A frequent approach for qPCR data normalization is the use of invariant endogenous controls where the gene expression levels are determined relative to a reference to standardize the amount of RNA added to the reaction. This internal control, often referred to as a housekeeping gene, should not vary in the tissues or cells under investigation. But the literature shows that housekeeping gene expression, although occasionally constant in a given cell type or experimental condition, can vary considerably. Genes involved in the maintenance of the cell, believed to be expressed at approximately constant levels are frequently used. In paper I, the average value of the genes β-2-microglobulin (*B2M*), TATA box binding protein (*TBP*) and eukaryotic 18S rRNA (*18S*) was used for normalization, while glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) was used in paper III. *GAPDH* mRNA levels have been shown to modulate under many circumstances both *in vitro* and *in vivo*, however in paper III *GAPDH* was only used to compare untreated and 5-Aza treated cell lines, and no comparison across sample groups were done (Suzuki et al. 2000).

The software NormFinder identifies the optimal normalization gene among a set of candidates. The algorithm is based on a mathematical model of the gene expression, and is used for estimating variation in the dataset. The intra- and inter-group variations are estimated separately and thereafter combined into a stability measure representing the estimated systematic error; a low stability value means a low systematic error and thereby a stable expression across samples. For the miRNA assays, small nuclear RNAs (snRNAs) involved in

maintaining processes in the cells, were used as references. Both RNU6B and RNU44 were tested as endogenous controls for calibration of the miRNA qRT-PCR data in paper II. Using Normfinder, the combination of RNU6B and RNU44 gave the lowest (best) stability value, accounting for the intra- and inter-group variation between the clinical osteosarcoma samples, osteoblasts and bone. However, since RNU6B and RNU44 followed the same expression pattern only RNU44 was used as a reference gene in paper III.

In paper I, qRT-PCR was used in order to validate the expression level of *CXCR4*. The same samples were included in the initial microarray analyses and the qRT-PCR experiment. The relative expression levels were in general similar using the two methods, with higher expression of *CXCR4* in the primary samples that developed metastases and in the metastatic samples. However, the relative differences in expression between the sample groups were smaller in the qRT-PCR data than the microarray data, and for the qRT-PCR data the expression level was not significantly different between the groups. In paper II, the qRT-PCR experiments performed on the clinical samples confirm the *in vitro* findings from the microarray experiments as 13/15 miRNAs showed the same expression pattern in clinical samples compared to bone as in the cell lines. Thus, although the data were generated using different methods (microarray vs. qRT-PCR) the findings were validated.

### miRNA target prediction

Following the initial identification of deregulated miRNAs (paper II), we predicted the potential target genes of these consistently up- or down-regulated miRNAs. Multiple alternative target prediction algorithms and online databases are available. The algorithms based on conservation criteria include TargetScan (Lewis et al. 2005), miRanda (Enright et al. 2003; Betel et al. 2008) and DIANA-MicroT (Kiriakidou et al. 2004; Reczko et al. 2012). PITA (Kertesz et al. 2007) and rna22 (Miranda et al. 2006) belong to the algorithms using other parameters such as free energy of binding or secondary structures of 3' UTRs that can promote or prevent miRNA binding (reviewed in (Witkos et al. 2011). The predictions can be used in combination with the manually curated experimental target database TarBase (Vergoulis et al. 2012). A target predicted by more than one program may be assumed to be more accurate, but in most cases an accurate algorithm is better as many of the combinations perform worse than the prediction of a single algorithm. The reason is that the combinations

of algorithms may result in an increase of the sensitivity at the cost of specificity (Alexiou et al. 2009).

TargetScan, used for target prediction in paper II, has been considered among the best performing databases for target prediction in several studies (Selbach et al. 2008; Maragkakis et al. 2009; Wang and Li 2009) and correlates well with experimental protein data (Baek et al. 2008; Selbach et al. 2008), and was. TargetScan predicts targets of miRNAs by searching for the presence of phylogenetically conserved 7mer and 8mer sites that match the seed region of each miRNA (Friedman et al. 2009). Typically, it is believed that a binding of at least 7 consecutive Watson-Crick base pairing nucleotides between the miRNA seed sequence and the mRNA is required for sufficient mRNA destabilization or repression of protein production. In TargetScan, target predictions can be limited to only miRNA sites that are phylogenetically conserved, as defined by the measurements of evolutionary relationship based on conservation of branch length in a phylogenetic tree (Kheradpour et al. 2007). In paper II, the targets were ranked based on the level of conservation of each of the miRNA target sites among vertebrates or mammals; a probability of preferentially conserved targeting ($P_{CT}$) value has been calculated for all highly conserved miRNA families, where the $P_{CT}$ value reflects the probability that a site is conserved due to selective maintenance of miRNA targeting rather than by chance or any other reason not pertinent to miRNA targeting (Friedman et al. 2009).

### Integration of different microarray data sets

To characterize how miRNAs may regulate their target mRNAs (paper II), we performed an analysis on the correlated expression of miRNAs and their putative target mRNAs. Several lines of evidence suggest that elevation of miRNA expression leads to downregulation of the target genes, and *vice versa* reduction of miRNA expression leads to upregulation of the target genes. Pearson's correlation (*r*) was calculated for each miRNA and its predicted mRNA targets across all the osteosarcoma cell line samples. Only data for osteosarcoma cell lines were used as bone showed an expression pattern different from the cell lines causing too high correlations to be reported. 116 mRNAs were predicted targets of identified miRNAs, were significantly differently expressed in both osteosarcoma cell lines and clinical samples versus normal bone, and showed a negative correlation with the miRNA in question. miRNA/mRNA

pairs with $P_{CT}$ <0.4 were then removed from the dataset, reducing the mRNA targets to 72. Finally, 26 mRNAs that showed an inverse correlated expression pattern with at least two miRNAs were presented.

The above analysis included several stringent filtering steps at both the miRNA and mRNA level. Only miRNAs belonging to evolutionarily conserved families (Friedman et al. 2009), indicating functional conservation and important roles, were included in the analysis, but there is also an abundance of miRNAs that are not conserved and may play a role in target regulation. Frequently, multiple sites either for the same or different miRNAs are associated with greater mRNA destabilization (Brennecke et al. 2005). A high cooperativity of two miRNA target sites have been observed when the sites are close to each other (Grimson et al. 2007; Saetrom et al. 2007). However, this distance metric was not taken into account in our last filtering step. It is important to bear in mind that miRNAs may repress their target genes only at the protein level and the targets may not exhibit noticeable changes at the mRNA level.

In order to identify epigenetically regulated miRNAs, miRNA expression levels were integrated with the methylation levels of associated upstream CpG sites in osteosarcoma cell lines, normal bone and osteoblasts (paper III). Seventy miRNAs that showed significant methylation (DeltaBeta>0.4) between osteosarcoma and normal samples were identified. In addition, Pearson's correlation (r) between the methylation level (avgBeta) of associated upstream CpG sites and miRNA expression level was calculated, identifying additional 20 miRNAs that showed anti-correlation between expression and DNA methylation (r <-0.5). The correlation was calculated across the cell lines only and in the panel of cell lines, bone and osteoblasts. The analysis performed in only the cell lines added just four new miRNAs as most of these had already been identified through the DeltaBeta analysis.

Identification of putative target genes was done by calculating the Pearson's correlation between miRNA and mRNA data (paper II). A similar approach for identifying target genes was used in a study of the NCI cell line panelwhere Pearson's correlation was calculated between miRNA and mRNA expression profiles. In this study, the correlation coefficients

were filtered based on a p-value (Wang et al. 2009). In our experimental set-up, both the miRNA and mRNA datasets was initially filtered based on p-value. A second t-test should not follow an initial t-test and a p-value filtering on the correlation level was not performed. In both paper II and III, Spearman's correlation could alternatively have been used on the dataset. While Pearson's correlation assumes a parametric linear correlation and normally distributed data, Spearman's rank-order correlation is non-parametric and suited for non-normally distributed data. When the sample size is small (<5) or there are outliers in the expression data, this correlation may be more reliable. In paper III, both Pearson's and Spearman's correlation were initially calculated, and the calculated correlation was generally lower when using Spearman's correlation.

### Validation of methylation

5-aza-2´-deoxycytidine

In paper III, we treated osteosarcoma cell lines with 5'-Aza in order to activate methylated genes. 5'-Aza is a cytosine analogue which is incorporated into the DNA and forms an irreversible covalent complex with DNMT1 (Santi et al. 1983; Christman 2002). This will in turn lead to depletion of DNMT1 in the cell during DNA replication, causing a passive demethylation and subsequent reactivation of genes (Velicescu et al. 2002). It has been reported that 5-Aza is highly effective not only at removing cytosine methylation but also at rapidly reversing chromatin structural changes (Nguyen et al. 2002). To achieve a successful effect, 5'-Aza must be administered to dividing cells, and the agent must be active through successive rounds of DNA replication. 5'-Aza is cytotoxic, and low concentrations not killing the cells should be administered. Initially, 1 μM and 10 μM concentrations of 5'-Aza were administered to the cells, but 1μM was observed to be most effective. Six of the nine candidate miRNAs, as well as miR-34a, displayed increased expression after treatment with 5'-Aza in at least two of the cell lines. The combinational treatment of 5'-Aza with a histone deacetylase inhibitor (e.g. trichostatin A) have been shown to cause synergistic reactivation of silenced genes in cancer cell lines (Cameron et al. 1999), and might have increased the efficiency of the treatment in our study.

*Sodium bisulfite treatment for modification of DNA*

DNA modification by the use of sodium bisulfite treatment has become the standard for DNA methylation analysis. This method can define the methylation state of every cytosine residue in the target sequence, at single-molecule resolution. The technique is based on a chemical process where sodium bisulfite deaminates cytosine into uracil, but does not affect 5-methylcytosine, a methylated form of cytosine with a methyl group attached to carbon 5 (Frommer et al. 1992; Clark et al. 1994). During DNA sequencing, the cytosines that are read as cytosines represent methylated cytosines, while those that are read as thymines represent unmethylated cytosines in the genomic DNA. Thus, an efficient rate of conversion of unmethylated cytosine to uracil is critical for the correct interpretation of the following DNA methylation analysis. High quality DNA and optimal experimental conditions are crucial for a complete DNA modification (Grunau et al. 2001; Clark et al. 2006). The majority of data on DNA methylation are based on prior treatment of the DNA with bisulfite, followed by DNA amplification with target specific primers. The detection techniques include pyrosequencing, bisulfite restriction analysis (COBRA), full genome bisulfite sequencing, methylation arrays, direct bisulfite sequencing, MSP and qMSP, of which the last four methods were used in this thesis and are discussed below.

*Methylation-specific PCR*

After bisulfite treatment, the methylation pattern of relevant CpG sites can be studied using MSP. This method is particularly useful to interrogate CpG islands with possibly high methylation density. MSP is a sensitive, semi-quantitative detection method where the amplification is performed with primers specific for methylated versus unmethylated sequences, enabling the identification of regions that are fully methylated, unmethylated or partially methylated. The methylation-specific primer pair is designed to bind only unconverted 5-methylcytosines covered by the primers, and conversely the unmethylated primer set (covering the same sites) specific complements thymines converted from unmethylated cytosines (Herman et al. 1996). If conversion is incomplete, the unconverted unmethylated cytosines will incorrectly be interpreted as methylated cytosines in the subsequent analyses, resulting in false positives. Furthermore, the primer design is critical. One should ensure that the primers anneal with a part of the promoter relevant for expression of the downstream gene. In paper III, the primers for *mir-486* were designed to complement

CpG islands close to transcription start site (TSS) as the methylation status of CpG sites in the area close proximity to TSS are assumed to be more likely to affect gene expression than sites far upstream of the promoter. To increase the specificity of the assay, the primers included multiple CpG sites and the additional presence of non-CpG cytosines avoided amplification of unmethylated unconverted DNA.

The PCR products from the MSP were separated by gel electrophoresis and stained with ethidium bromide, and visually scored according to three previously described sample categories, strongly methylated, weakly methylated and unmethylated (Smith-Sorensen et al. 2002). Bisulfite treated normal blood and human placental DNA treated *in vitro* with SssI methyltransferase were used as positive controls for unmethylated and methylated reactions, respectively. In brief, samples with equal or stronger band intensity than the positive control in the methylation-specific reaction were denoted strongly methylated. Samples with less intense bands than the positive control were categorized as weakly methylated, whereas samples with very weak or no visible PCR product in the methylated reaction and a band in the unmethylated reaction were regarded as unmethylated. To ensure correct interpretation, the MSP data in paper III were scored by two independent investigators.

*Bisulfite sequencing*

While MSP provides a score of methylated or unmethylated for a region of interest, bisulfite sequencing can be used to give a more accurate picture of the methylation pattern at base pair resolution. Unmethylated cytosines appear as thymines after bisulfite modification, while 5-methylcytosines appear as cytosines in the final sequence (Clark et al. 1994). Bisulfite sequencing is performed to verify the methylation statuses from the initial and rapid MSP analysis, and to confirm whether or not the MSP assay is designed to amplify a representative region of the gene promoter. The primers for bisulfite sequencing are designed to amplify both methylated and unmethylated alleles. Primers designed outside of a CpG region of interest will, in principle, amplify the target regardless of the methylation state of the internal sequence. Both direct and cloning-based bisulfite sequencing can be performed. For paper III, a direct, semi-quantitative sequencing was performed on the cell lines, resulting in an average value of methylation. Alternatively, the amplified PCR product can be cloned into plasmid vectors followed by sequencing of the individual clones. The level of detail obtained from

clonal analysis highlights the degree of methylation heterogeneity that is often observed in clinical samples, and this detail is often lost using direct PCR sequencing (Clark et al. 2006).

*Quantitative methylation-specific PCR*

qMSP is a high throughput methylation assay that utilizes fluorescence-based real-time PCR (e.g. TaqMan) technology to quantitatively determine the relative prevalence of a particular pattern of DNA methylation. qMSP is a highly sensitive assay, capable of detecting methylated alleles in the presence of a 10,000-fold excess of unmethylated alleles (Eads et al. 2000). qMSP is a high throughput method ideally suited for the analysis of large numbers of clinical samples. However, qMSP is a stringent method where both primers and probe are specific for fully methylated DNA, and partially methylated target sequences will not be amplified. Thus, the methylation level may be higher than those determined.

*Methylation arrays*

The advances in bisulfite methods have led to the possibility of performing analyses at a genome-wide scale. For paper III, two different methylation arrays from Illumina were used. The Infinium HumanMethylation27 BeadChip quantitatively interrogate >27,000 CpG sites located within the proximal promoter regions of transcription start sites of >14,000 genes at single-nucleotide resolution. In addition, 254 assays cover 110 miRNA associated promoters. Although a high number of genes are measured, on average, only two CpG sites are assayed per locus, but with a higher coverage (3-20 CpG sites) for cancer-related and known imprinted genes. The CpG sites are mainly located in the promoter CGIs. The Infinium HumanMethylation450 BeadChip covers >485,000 CpG sites across the genome and includes > 90% of the 27k array content with an average of 17 CpG sites per gene region. In addition to CpGs located in CGIs, CpG sites on the island shores and shelves, first exon and gene body, outside of coding regions (5' UTR, 3' UTR), non-coding RNAs (miRNAs and long non-coding RNAs) and non-CpG methylated sites identified in human stem cells are included on the array.

The quantification method is based on gene-specific and methylation-dependent single-nucleotide primer extension on bisulfite-converted DNA. The 27k assay design employs two

bead types per CpG locus and use primers that distinguish between unmethylated (U bead type) and methylated (M bead type) CpGs after bisulfite conversion. For the probes unique to the 450k design, one bead type is used with the methylation stage determined at the single base extension step after hybridization. For both assays, the level of methylation for the interrogated locus can be determined by calculating the ratio of the fluorescent signals from the methylated versus unmethylated sites.

## Discussion of results

### A metastatic expression profile in osteosarcoma

Osteosarcoma invasion of bone relies on interactions between the bone matrix, osteosarcoma cells, osteoblasts and osteoclasts. The relative activities of osteoblasts and osteoclasts are normally tightly coupled in order to maintain a balance between bone formation and degradation. Bone remodelling is regulated both by systemic hormones and locally produced cytokines. Cells in the bone marrow, especially stromal and immune cells, produce cytokines and growth factors that influence the activities of osteoblasts and osteoclasts. However, this balance between bone synthesis and resorption is disturbed in several pathological conditions, including osteoporosis, rheumatoid arthritis and skeletal metastases. Continuous and dynamic bone matrix and bone marrow cell turnover provide fertile ground for the homing and subsequent proliferation of tumour cells, reviewed by (Bussard et al. 2008).

Recurrence of osteosarcoma usually occurs as pulmonary metastases or, less frequently, as metastases to distant bones or a local recurrence (Kempf-Bielack et al. 2005; PosthumaDeBoer et al. 2011). Thus, a novel strategy that would efficiently inhibit metastasis, especially to the lung, is highly desirable. It has been shown that already at primary diagnosis, a very high fraction of osteosarcoma patients have micrometastatic cells in bone marrow, and a correlation between the presence of tumour cells, clinical stage, and disease progression have been found (Bruland et al. 2005), making it possible to identify patients with an increased risk of metastatic disease. In paper I, the differences in gene expression pattern between primary and metastatic osteosarcoma were investigated. In general, genes belonging to pathways involved in immunological processes and chemokine and cytokine signalling were higher expressed in metastases compared to primary samples. A comparison was also done between primary samples of patients that had or had not developed metastases, and genes that showed different expression levels between these two groups were identified.

#### *CXCR4 expression in primary tumour cells*

In paper I, the expression level of *CXCR4* was associated with subsequent development of metastases in osteosarcoma; primary samples from patients with a metastatic disease showed higher levels of *CXCR4* expression than those without metastases, comparable to the

expression level in metastases. The gene expression analysis has been performed using bulk tumour tissue, and the profiles represent the average profile of the entire tumour. Thus, the presence of a metastatic pattern indicates that a metastatic potential is an inherent capacity of the majority of the cells in the primary tumour.

The osteosarcoma tissue contains both tumour cells as well as infiltrating mononuclear cells (Theoleyre et al. 2005), and the interactions between these cell types are critical in the metastasis formation. In our study, high expression of *CXCR4* was observed in patient samples, but not in xenografts and cell lines (not shown), suggesting that chemokine expression is dependent on the human stromal cells. It has previously been argued for a specific role of CXCR4 expressed in stromal cells that conditioned the pro-tumour microenvironment (D'Alterio et al. 2012). A recent study compared the gene expression profile of osteosarcoma patients who did or did not develop metastasis. Genes with immunological functions, particularly related to macrophages, were overrepresented among the upregulated genes. The tumour cells and their stromal context were further investigated, and it was shown that the macrophage-associated genes were expressed by infiltrating cells and not by osteosarcoma cells, and it was unexpectedly postulated that the presence of macrophages was associated with reduced metastasis and improved survival in high-grade osteosarcoma, in contrast to most other tumour types (Buddingh et al. 2011).

### *CXCR4 and VEGF expression in primary and metastatic samples, and the prognostic potential of CXCR4*

The interaction between the chemokine receptor CXCR4 and its ligand CXCL12 attracts cancer cells to leave the circulation and migrate into organs with large amounts of chemokines. The CXCR4/CXCL12-axis has been found to play an important role in tumourigenicity, proliferation, metastasis, and angiogenesis in cancer (Muller et al. 2001; Perissinotto et al. 2005; Chen et al. 2006; Ehtesham et al. 2009). CXCL12 is expressed at high levels in the lung, which is the primary site to which osteosarcoma tumours metastasize. No correlation was observed between the level of *CXCR4* and *CXCL12* in primary osteosarcoma (paper I, not shown). Limited sample number severely limits the power of the study, but to our knowledge it represents the first study where an association between *CXCR4* and metastasis has been identified using microarray experiments.

CXCR4 has also been linked to other genes associated with a metastatic potential in osteosarcoma, like the vascular endothelial growth factor (VEGF). VEGF is a critical mediator of angiogenesis and tumour proliferation (Hicklin and Ellis 2005). In our study, *CXCR4* and *VEGF* showed a correlated expression pattern in primary samples (Pearsons' correlation r=0.60). Similar to what was observed with *CXCR4*, *VEGF* was expressed at the same level in metastatic samples and in primary samples that developed metastases. The expression level was significantly lower in primary samples that did not metastasize compared to the two other groups (Mann-Whitney test, p=0.02 for both comparisons, Figure 8).

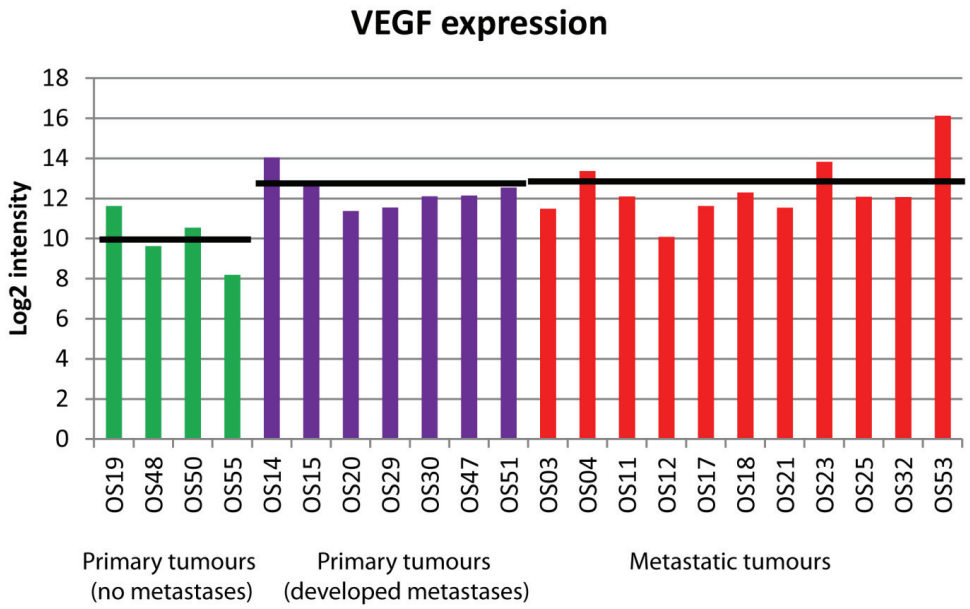


**Figure 8.** Relative *VEGF* expression in primary tumours from patients who did or did not develop metastases and in metastatic samples based on microarray data. The average expression level in each sample group is indicated with a black line.

Using immunohistochemistry, Oda et al. (Oda et al. 2006) compared immunohistochemical expression level of CXCR4 and VEGF at primary and metastatic sites in 30 osteosarcoma patients. CXCR4 expression at the metastatic site was significantly increased compared with that at the primary site, whereas no difference between primary and metastatic sites was observed with regard to VEGF expression. However, there was a significant positive correlation between CXCR4 and VEGF expression; that is, samples were more likely to have both proteins expressed. Moreover, the patients with VEGF-positive primary tumours had a

significantly worse prognosis than did patients with VEGF-negative primary tumours, while no relationship between CXCR4 and survival was seen. A recent study by Lin et al reported immunohistochemical expression of both CXCR4 and VEGF to be correlated with poor survival. In line with our findings, primary tumour tissues from patients that were presented with or developed metastases were more likely to be positive for CXCR4 than patients with non-metastatic disease (Lin et al. 2011). The same observation was seen for VEGF. An association between *CXCR4* mRNA level and survival was reported by Laverdiere et al. showing an inverse correlation with both the presence of metastases at diagnosis and survival (Laverdiere et al. 2005). Furthermore, higher CXCR4 mRNA and protein level in high-grade compared to low-grade osteosarcoma has been observed (Laverdiere et al. 2005; Lin et al. 2011). Contradicting the above observations, Baumhoer et al. investigated the potential role of CXCR4 and CXCL12 in an extensive panel of 223 untreated osteosarcoma samples, and found these chemokines to correlate with a better long-term outcome and with a lower prevalence of metastases, although the correlations only met a significance level for CXCL12 (Baumhoer et al. 2012a). The observed differences of the prognostic value of CXCR4 could partly be explained by a difference in scoring criteria and the choice of antibodies, as the monoclonal antibody most commonly used to study CXCR4 expression, 12G5, recognizes only a sub fraction of CXCR4 molecules (Baribaud et al. 2001). CXCR4 is a membrane-bound receptor that, upon activation, is internalized into the cytoplasm. Only nuclear and cytoplasmic staining was evaluated in several of the studies, while Baumhoer et al also scored CXCR4 in membranous compartments.

### *CXCR4 and the metastatic process in bone*

While the primary metastatic site for osteosarcoma is the lung, breast cancer metastasis shows a tropism for bone and secondary to lung, liver and lymph nodes. Metastasis to bone is thought to be influenced by CXCL12 signalling. CXCL12 is abundantly expressed by osteoblasts and bone marrow stromal cells, and immunohistochemistry of developing bones demonstrated that CXCL12 expression was a feature of early bone development (Jung et al. 2006). The impact of CXCR4 on the metastatic process in breast cancer has been thoroughly investigated in numerous studies. Neutralization of CXCR4 or CXCL12 significantly impaired migration of breast cancer cells, and anti-CXCR4 caused a reduction in lung metastases in an *in vivo* model (Muller et al. 2001). As for osteosarcoma, the prognostic value of CXCR4 varied between the different studies, and one reason for these observations may be

that different molecular subclasses of breast cancers, like human epithelial growth factor receptor 2 (HER2) and estrogen receptor (ER) positive and negative tumours, are included in the different studies. It has been shown that HER2 enhances the expression of CXCR4, which is required for HER2-mediated invasion *in vitro* and lung metastasis *in vivo*. A significant correlation between HER2 and CXCR4 expression was observed in human breast tumour tissues, and CXCR4 expression correlated with a poor overall survival rate in patients with breast cancer (Li et al. 2004). A correlation between HER2 and CXCR4 was shown in high-grade osteosarcoma (Ma et al. 2012), although these findings should be further verified. In a study with an enrichment of ER positive disease, CXCR4 was not prognostic for overall survival, and was not associated with a higher risk of liver, brain or lung metastases. But CXCR4 expression in primary tumours was found to be associated with a higher risk for subsequent development of *bone* metastases in patients with early breast cancer (Andre et al. 2009). This suggests that there is a need for new biomarkers complementary to CXCR4 to accurately predict the occurrence of metastases. These findings also highlights an important aspect that must be carefully considered during the development and trial of new treatment therapies; there is a high degree of diversity between and within tumours as well as among cancer-bearing individuals, and all of these factors together determine the risk of disease progression and therapeutic resistance.

*Anti-CXCR4 therapy*

Therapy aimed at disruption of the specific CXCR4-receptor/CXCL12-ligand complex may lead to a decrease in metastases. Supporting this approach, development of lung metastases after injection of osteosarcoma cells into murine xenografts was prevented by the administration of small peptide CXCR4 inhibitors, T134 (Perissinotto et al. 2005) or CTCE-9908 (Kim et al. 2007). The first CXCR4 antagonist, Plerixafor (previously named AMD3100), is approved by FDA for use in mobilization of hematopoietic stem cells to peripheral blood. Several other CXCR4 inhibitors are in clinical development, such as the peptidic inhibitor CTCE-9908. The effect of CTCE-9908 has been evaluated in a small phase I/II study in solid tumours (Kavsak et al. 2009), and several clinical studies are ongoing in which newly developed CXCR4 antagonists are evaluated in hematological neoplasias as mobilizing agents (www.clinicaltrials.com). Thus, although the prognostic value of CXCR4 is debated in osteosarcoma, an functional effect of CXCR4 expression has been shown.

### A miRNA expression profile in osteosarcoma

In paper II, deregulated miRNAs in osteosarcoma were identified. Initially, only microarray data and qRT-PCR data of osteosarcoma samples and normal bone samples were included. miRNA microarray data for two human calvarial osteoblast cultures were available, but as no statistical analyses could be done on this set of only two samples we decided to rather base our global analyses on a comparison against the four bone samples. Towards the end of the work with this manuscript, a global expression profile describing deregulated miRNAs in osteosarcoma compared to osteoblasts was published (Maire et al. 2011). Among the list of 38 deregulated miRNAs identified by Maire et al., 16 miRNAs were also found to be deregulated in our cell lines compared to bone, and all but three miRNAs were found to be oppositely regulated in these two studies. In order to verify these observations, a smaller cohort of clinical samples, normal bone and osteoblasts was collected.

#### *The deregulation of identified miRNAs is consistent across different cohorts and analysis platforms*

qRT-PCR experiments were performed on a cohort of 12 clinical samples, six normal bone and five osteoblasts samples. The expression level of a set of 15 miRNAs, representing a subset of the most differently expressed miRNAs identified in our study (selected from Table 1 in paper II), was determined. Of these, seven miRNAs were commonly identified in both our study and the study from Maire et al. For these 15 miRNAs, miRNA expression levels from microarray data of osteosarcoma cell lines and normal samples are shown in Figure 9a and miRNA expression levels from qRT-PCR data for osteosarcoma clinical samples and normal samples are shown in Figure 9b. For most of the miRNAs, the real-time experiments performed on the clinical samples confirm our findings from microarray experiments on the *in vitro* cell lines, thus despite changes in samples and analysis, the expression patterns of the miRNAs are well maintained. This makes it unlikely that the deregulation of these miRNAs is an effect of *in vitro* cultivation or data normalization. Even though we cannot exclude that the normalization procedure could have some impact on the expression level of some of the miRNAs, we are confident that the overall findings are reliable and that the data describe the actual state of miRNA expression for the different groups of samples.

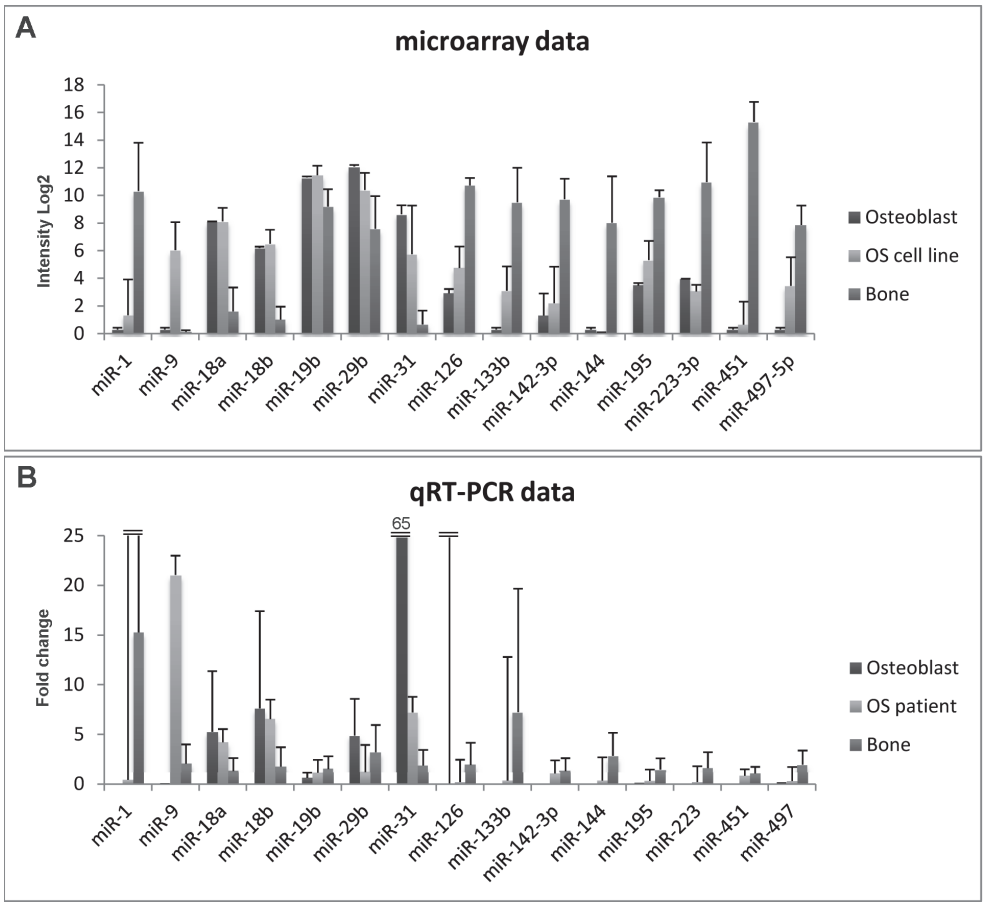


**Figure 9**. Expression level of 12 miRNAs in osteosarcoma and normal samples. **A**. miRNA expression in osteoblasts, osteosarcoma (OS) cell lines and bone based on microarray data. **B**. miRNA expression in osteoblasts, osteosarcoma (OS) patient samples and bone based on qRT-PCR data.

In our global analysis comparing osteosarcoma and bone, 177 miRNAs was identified as significantly differently expressed. Of the 34 miRNAs identified as deregulated by Jones et al. (Jones et al. 2012), 16 miRNAs were also significantly changed in our study. Similarly, 17 of the 36 miRNAs described by Thayanithy et al., (Thayanithy et al. 2012a) were confirmed to be deregulated in our study. Importantly, all but three of these miRNAs showed similar up/down-regulation in our study as previously described by Jones and Thayanithy. Thayanithy et al identified the expression level of miRNAs of the miR-17-92 and miR-14q32 clusters to be increased or decreased in osteosarcoma, respectively, compared to normal bone. All miR-17-92 miRNAs were significantly upregulated in our osteosarcoma cell lines compared to bone, and the upregulation of miR-18a was confirmed in clinical samples. All

seven 14q32 miRNAs (miR-127-3p, miR-134, miR-154, miR-382, miR-369-3p, miR-544 and miR-656) were lower expressed in our osteosarcoma cell lines than in bone, but the level of change was only significant for miR-134. Thus, despite having different cohorts, platforms and analysis pipelines, our findings are consistent with the previous publications in this field.

*miRNAs with intermediate expression level in osteosarcoma compared to osteoblast and bone*

Interestingly, all but two of the 15 miRNAs showed an intermediate expression level in osteosarcoma clinical samples compared to the mean values in osteoblasts and bone. These 13 miRNAs include the miRNAs miR-31, miR-126, miR-142-3p, miR-195, miR-223, miR-451 and miR-497 previously revealed in osteoblasts (Maire et al. 2011) as well as miR-1, miR-18a, miR-18b, miR-19b, miR-133b and miR-144. During the last year, several studies describing the expression profiles of osteosarcoma relative to either bone or osteoblasts have been published. However, in our study, data for both osteoblasts and bone have been included. The observed inverse regulation of miRNAs compared against bone or osteoblasts is consistent with the previous publications; Jones et al. (Jones et al. 2012) found miR-126, miR-142-5p, miR-195, miR-223 and miR-451 to be downregulated in osteosarcoma versus bone, while Lulla et al. (Lulla et al. 2011) reported a subset of these, miR-126, miR-142-3p, miR-223 and miR-451 to be upregulated compared to osteoblasts. miR-195 was recently shown to suppress osteosarcoma cell invasion and migration by targeting the fatty acid synthase (FASN) (Mao et al. 2012).

*Origin of osteosarcoma in the context of normal samples and miRNA expression*

Osteosarcomas are malignant neoplasms of mesenchymal origin, and unlike cancers of epithelial origin, osteosarcoma does not have an obvious multi-step progression. Low grade osteosarcomas are not believed to be a precursor entity of high grade osteosarcomas. Thus the pre-malignant lesion has not been investigated and the origin and developmental process leading to osteosarcoma formation are largely unknown. Bone organogenesis is a complex process involving the differentiation and crosstalk of multiple cell types for formation and remodelling of the skeleton. Bone tissue is continuously turned over and remodelled through the activities of two main cell populations (Lian et al. 2012). The bone-resorbing osteoclasts arise from the hematopoietic lineage and the bone-forming osteoblasts are of mesenchymal

origin (Figure 10). The osteosarcoma tumour cells produce osteoid, or tumourous bone, and this has led to the assumption that osteosarcoma arise from osteoblastic cell-lineages. Conventional osteosarcomas are divided into different histological subtypes, and may display chondroblastic, fibroblastic and osteoblastic components. There is no evidence that osteoblasts, once they differentiate from osteoprogenitor cells, can actually revert into more primitive and malignant cells, and this suggests that the cell of origin is more pluripotent than osteoblasts (Klein et al. 2006). Mesenchymal stem cells are potentially the cells of origin given their multipotential differentiation capacity, and osteosarcoma may develop as result of genetic changes occurring during the determination or maturation of mesenchymal stem cells or committed progenitor cells (reviewed in (Mohseny and Hogendoorn 2011a)). Since most osteosarcomas have a fully induced bone-forming phenotype, it seems conceivable that they, in several regards, are as differentiated as the normal osteoblasts. However, their increased proliferation and scrambled genome preclude normal differentiation and promotes disorganized growth. In our study, osteoblasts and normal bone cells represent the immature and fully mature endpoints of bone differentiation, respectively, both representing normal samples. The observed differences between osteosarcoma, bone and osteoblasts in our study may both be related to the differentiation level and to the malignant process of osteosarcoma development.

Inhibition of mRNA translation by miRNAs has emerged as an important regulator of osteogenesis. *In vivo* studies in mice show that Dicer generated miRNAs are essential for two periods of bone formation, to promote osteoblast differentiation before birth, and to regulate bone mass in adult mice (Gaur et al. 2010). Thus, during bone formation, it is likely that distinct sets of mature miRNAs contribute to every step of osteogenesis by regulating the progressive differentiation of the cells that constitute bone tissue by repressing modulators of pathways operating in these cells. The miRNA expression pattern observed in osteosarcoma may reflect a specific stage of differentiation. By improving our knowledge of the regulatory circuits controlled by individual miRNAs and the roles of the many miRNAs that are responsive to and regulate osteogenic signals in bone, we will increase the understanding of the mechanisms involved in osteogenesis and the developmental processes of osteosarcoma.

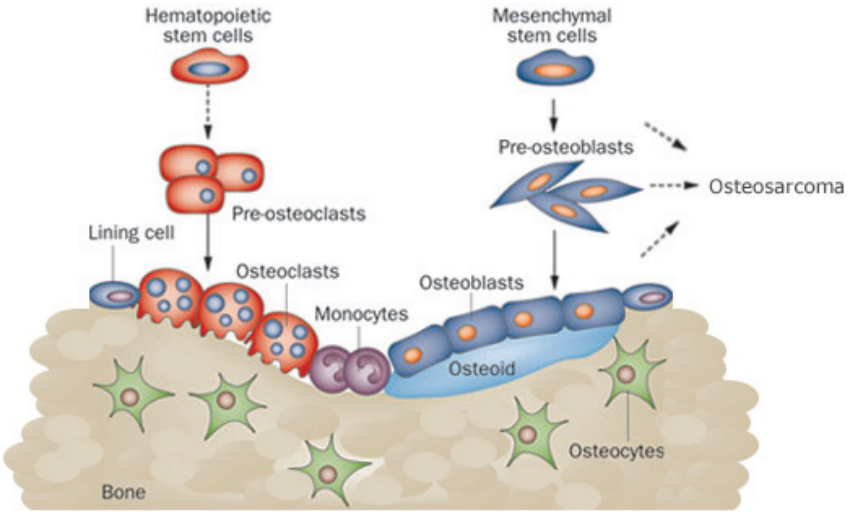


**Figure 10.** The bone remodelling cycle and development of osteosarcoma. Osteoclasts originate from hematopoietic stem cells which differentiate to a committed mononuclear preosteoclast cell that fuses to form the multinucleated osteoclast cells. Attachment of osteoclasts erode bone surfaces, and debris is removed by monocytes or macrophages. Osteoblasts originate from mesenchymal stem cells which differentiate to pre-osteoblasts and further to mature osteoblasts. In the bone formation phase the pre-osteoblasts replace the resorbed bone surfaces and osteoblasts produce osteoid matrix which will mineralize. Osteosarcoma may arise from mesenchymal stem cells or during the differentiation of osteoblasts. Adapted from (Lian et al. 2012).

### Epigenetic regulation of miRNAs in osteosarcoma

Epigenetic changes, as altered DNA methylation, cause deregulated miRNA expression in cancer (reviewed by (Iorio et al. 2012)). Few studies are done on epigenetic regulation on miRNAs in osteosarcoma, but He et al., has shown that the expression levels of miR-34b and miR34c was lower in primary osteosarcoma samples than in adjacent normal tissues, where both loss of heterozygosity and methylation were observed among the primary tumours (He et al. 2009). In addition, the miRNAs in the 14q32 locus were recently shown to be epigenetically regulated in human and canine osteosarcoma cell lines. A combination of the DNA and chromatin-modifying drugs 5-Aza and 4-phenylbutyric acid (PBA) caused histone deacetylation and activation of the 14q32 miRNAs, but DNA methylation did not seem to be a primary factor for downregulation of these miRNAs in human osteosarcoma. Treatment of Saos-2 cells induced pro-apoptotic genes and downregulated the expression level of cell cycle genes, and showed a functional effect by induction of apoptosis. Finally, the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) and the DNA methylation inhibitor zebularine, both of which are approved for clinical use, showed additive cytotoxicity

in human and in canine osteosarcoma cells with aggressive biological behaviour (Thayanithy et al. 2012a).

*Global methylation pattern of osteosarcoma and normal samples*

For the 450k methylation arrays used in the confirmation experiments in paper III, 12 osteosarcoma patient samples were initially included. After pathological revision, two of the samples, OS93 and OS94-1, showed to have less than 10% tumour content, and were removed from the study. Based on the global methylation pattern, OS93 behaved as an outlier compared to the other samples, while OS94-1 intermingled with the other patient samples (not shown). The osteosarcoma and normal samples clustered in two main subclusters, one with only patient samples and one with osteosarcoma patient samples and normal samples. The osteoblasts, being *in vitro* samples, diverged more from the patient samples than did the bone samples. One subgroup of the patients consisting of OS14, OS29, and OS88 were more similar to the group of normal samples than the rest of the osteosarcoma samples. However, no clinopathological features separated these samples from the remaining specimens (paper III, supplementary file 8). One patient sample, OS86-2, clustered inbetween bone samples although pathological revision estimated a proportion of tumour cells >70%. OS86-2 originated from one of the two patients included in the study reported to have no evidence of disease after the end of treatment.

*Epigenetic regulation of initial candidate miRNAs*

For the nine miRNAs initially identified in paper III, we generally observed high methylation in the cell lines and low methylation levels of both the normal bone and osteoblast samples. Treatment of osteosarcoma cells with the epigenetic modifier 5-Aza, resulted in increased expression of six of nine miRNAs; miR-224-5p, miR-452-5p, miR-34b-5p, miR-34c-5p, miR-335-5p and miR-486-5p. The candidate miRNAs miR-126-3p, miR-miR-598 and miR-631 were discarded after an initial screening as they were either undetected before and after 5-Aza treatment or did not respond to treatment at all. It has been shown that miR-126 was slightly upregulated after treatment with either 5-Aza or the HDAC inhibitor PBA alone in human cancer cells, but strongly upregulated after treatment with both 5-Aza and PBA (Saito et al. 2009). miR-224-5p and miR-452-5p showed increased expression in 2/7 and 3/12 cell lines, respectively, but this frequency was regarded as too low, and detailed characterization of the

upstream CGIs was not carried out. miR-224 expression was recently shown to increase after treatment with the HDAC inhibitor drugs trichostatin A (TSA) or SAHA in immortalized human liver cells, but not 5-Aza (Wang et al. 2012b). These results suggest that the CpG island promoters of these miRNAs may be regulated by chromatin structural changes such as histone modifications rather than by DNA methylation only in cancer cells.

*The consistency between methylation and expression level and effect of 5-Aza treatment*

For miR-34b-5p, miR-335-5p and miR-486-5p-5p, methylation data from MSP, bisulfite sequencing, qMSP and 27k methylation arrays, as well as miRNA expression from microarrays and qRT-PCR experiments were available for 12 osteosarcoma cell lines. For miR-34b-5p, the correlation between miRNA expression level and DNA methylation level, and the effect of 5-Aza treatment was consistent across all 12 investigated cell lines for all the analyses performed. Also for miR-335-5p, a clear relation was observed between the methylation and expression level for 10 of the cell lines, but IOR/OS18 and IOR/MOS did not show any effect of 5-Aza treatment. The investigated CGI of *mir-486* showed DNA methylation in all cell lines except IOR/OS14 as determined by the MSP and methylation arrays. miR-486-5p, although low expressed, had a higher basal expression level than what was observed for miR-34b-5p and miR-335-5p, and only showed a modest increase in expression upon 5-Aza treatment.

*miR-34 family*

The well known tumour suppressors of the miR-34 family, comprising *mir-34a*, *mir-34b* and *mir-34c*, have been shown to be lower expressed in primary osteosarcoma samples than in adjacent normal tissues (He et al. 2009). The members of the miR-34 family have been shown to be inactivated through epigenetic regulation in a range of solid tumours, including soft-tissue sarcomas (Toyota et al. 2008a; Vogt et al. 2011).

*mir-34a*, is located in a different locus than *mir-34b/c*. As the CGI is located >10 kb upstream of the miRNA, miR-34a was not identified in the initial analysis. However, miR-34a has been shown to be inactivated through epigenetic regulation (Vogt et al. 2011), and was therefore investigated in our study, although not included in paper III. miR-34a-5p showed from 2 to

10-fold induction in four of the 12 tested cell lines after 5-Aza treatment. The CGI upstream of *mir-34a* was hypomethylated in all patient samples as observed from the 450k methylation arrays.

*mir-34b/c* and B-cell translocation gene 4 (*BTG4*), believed to share a common bidirectional promoter, are frequent targets of epigenetic silencing in colorectal cancers (Toyota et al. 2008b). The expression of *BTG4* was undetected in the majority of the osteosarcoma cell lines both before and after 5-Aza treatment. A two-fold increase of *BTG4* was seen for 2/12 cell lines and miR-34b-5p was increased in 6/12 cell lines, but no association between *BTG4* and miR-34b-5p expression was observed. Fifty percent of the osteosarcoma cell lines and 17% of the xenografts were hypermethylated as determined by qMSP analysis. No methylation was measured for the tumour samples based on the qMSP data. Only one of the 10 patient samples showed a modest level of methylation based on the 450k methylation arrays. Previous published data reported methylation of the miR-34b/c locus in 15% of osteosarcoma tumours (He et al. 2009). The consistent patterns between methylation level, miRNA expression level and effect of 5-Aza treatment indicate that *miR-34b/c* are epigenetically regulated in the osteosarcoma cell lines, but the lack of methylation in patient samples indicate that this may be an *in vitro* bias. Further studies need to be performed with a larger panel of patient samples.

The miR-34 family has been identified as direct transcriptional targets of p53 (He et al. 2007). The function of miR-34a, miR-34b and miR-34c was investigated in the osteosarcoma cell lines U-2 OS and SAOS-2, showing that the basal expression levels of these miRNAs were p53-independent, while the miR-34s inhibited proliferation and induced G1 arrest and apoptosis in a partially p53-dependent manner (He et al. 2009). This is in agreement with our data, which showed that ectopic expression of miR-34c-5p reduced proliferation in the cell line MG-63. The expression of miR-34c has been shown to be increased during BMP2-mediated osteoblast differentiation. Transgenic mice overexpressing miR-34c had an imbalanced bone homeostasis due to reduced differentiation of osteoblasts and elevated osteoclastogenesis (Bae et al. 2012). miR-34c has been shown to target the osteogenic master regulator *RUNX2* (Zhang et al. 2011b) as well as Notch signalling (Bae et al. 2012), which may be important during the pathogenesis of osteosarcoma (Engin and Lee 2010).

*mir-486*

*mir-486* showed cancer specific methylation patterns in osteosarcoma cell lines, xenografts and patient samples using qMSP. In general, only minor differences between osteosarcoma primary tumours and normal samples were revealed in the Illumina 450k methylation data, but the CGI profiled with the qMSP showed higher changes in the 450k data. The qMSP method provided a higher density of a smaller, defined region, and the results from the 450k arrays do not exclude *mir-486* as a candidate epigenetically regulated miRNA. miR-486-5p were identified as highly downregulated in osteosarcoma in paper II, and has previously and shown to be consistently downregulated in cancer (Navon et al., 2009). Ectopic expression of miR-486-5p reduced cell proliferation in osteosarcoma cell lines, showing that this miRNA is capable of regulating important functions of cancer cells. Supporting these findings, high expression levels of miR-486-3p was associated with significantly increased recurrence-free and overall survival in hepatocellular carcinomas (Huang et al. 2012). Thus, *mir-486* is an interesting candidate miRNA in osteosarcoma.

*mir-335*

The maternally imprinted *mir-335*/ *MEST* (mesoderm specific transcript homolog (mouse)) locus (Nishita et al. 1996; Kosaki et al. 2000) is associated with two different CGIs, covering the TSS of transcripts 1,3 and 4 of *MEST*, located proximal to miR-335-5p, and transcripts 2,5 and 6 of *MEST*, located more distal to miR-335-5p, respectively. For the Illumina 27k methylation arrays, 10 probes were located in the upstream region of miR-335-3p/5p. All probes showed high methylation in the osteosarcoma cell lines. Probe cg01888566 showed the most negative Pearsons' Correlation between miRNA expression level and the methylation level in cell lines and normal samples, and were located in the more distal CGI (paper III, supplementary table 5). However, as MSP analyses recently revealed that a region of the proximal CGI displayed an increase in methylation in metastatic relative to nonmetastatic MCM7 cells and to normal female genomic DNA, we chose to use the MSP primers published in this study (Png et al. 2011). We then did an extensive mapping of the proximal CGI in osteosarcoma cell lines, but when normal samples were included in the qMSP analysis it was shown that the normal samples and tumour samples were all highly methylated. Thus, we decided to use the 450k methylation arrays to analyse the methylation pattern in the mir-335 upstream region. On the array, 91 probes mapped to the upstream regions of miR-335, of which eight were located in the distal CGI and 50 in the proximal

CGI. The distal CGI showed to be higher methylated in the tumour samples compared to the normal bone samples. Some differences were also observed in the 5' part of the proximal CGI, while the methylation pattern in the 3' part of this island (downstream of TSS of MEST transcripts 1 and 4) was identical for tumour and normal samples. The MSP and qMSP primers covered a region that is similarly methylated in normal and tumour samples. Contrary to the bone samples, the osteoblasts showed a methylation level similar to the tumour samples. These results show that the methylation patterns are different for the two CGIs in osteosarcoma, and indicate that the epigenetic regulation of this locus is specific for different cancer types or for the metastatic process.

miR-335 has been shown to be involved in both osteogenic differentiation and cell proliferation (Figure 11), making it a highly relevant gene in the context of osteosarcoma. The Rb and p53 pathways are frequently deregulated in osteosarcoma. It has been shown that miR-335 directly controlled human RB1 (pRb/p105) expression through binding to the RB1 3' UTR in osteosarcoma cell lines. The alteration of Rb1 levels resulted in a compensatory activation of the p53 tumour suppressor pathway, and the further effect on the cancer cells was dependent on the p53 status. Transient transfection of miR-335 into the p53-deficient cell line MG-63 significantly accelerated proliferation (Scarola et al. 2010), consistent with our observations in paper III, while transfection of U-2 OS, carrying p53 wild-type alleles, significantly decreased proliferation. miR-335 levels were increased during DNA damage in U-2 OS cells through upregulation of *MEST* in a p53 dependent manner, establishing a positive feedback loop between miR-335 and p53. Thus, miR-335 balances the activities of the Rb and p53 tumour suppressor pathways, affecting the control of cell proliferation and transformation (Scarola et al. 2010). Upon malignant transformation of the human osteoblast cell line hFOB1.19, multiple imprinted genes showed to be aberrantly expressed, among these *MEST*, being downregulated during osteosarcoma transformation (Li et al. 2008), and most likely also miR-335. miR-335 is significantly downregulated during differentiation of human mesenchymal stem cells, targets RUNX2, and regulates cell proliferation and migration (Tome et al. 2010). In osteoblasts, miR-335-5p activated wingless-type MMTV integration site family (Wnt) signalling and promoted osteogenic differentiation by down-regulation of dickkopf 1 homolog (Xenopus laevis) (*DKK1*), and high expression levels of miR-335-5p indicated that miR-335 could be essential for promoting bone formation and regeneration (Zhang et al. 2011a). Taken together, miR-335 represents a promising tumour suppressor in

osteosarcoma, and the epigenetic regulation of miR-335, as observed in our study, may contribute to the understanding of the development of this cancer type.

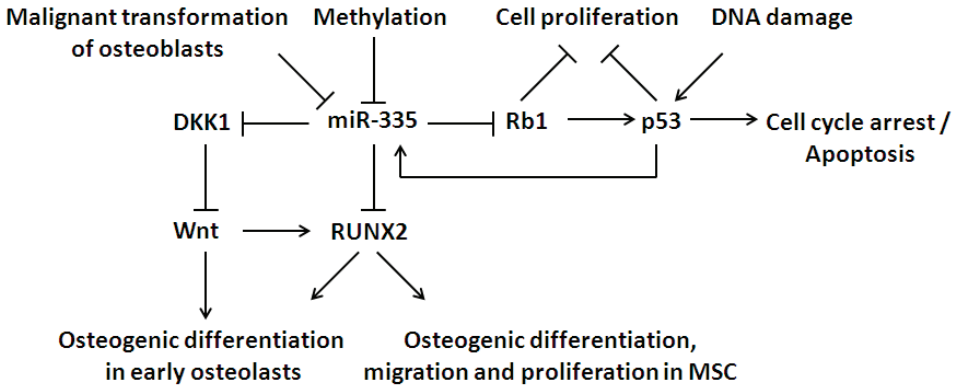


**Figure 11**. A model for miR-335 function during osteogenic differentiation, transformation and cell proliferation. miR-335 directly controls Rb1 which activates the p53 tumour pathway and upregulates miR-335 in a positive feedback loop. miR-335 regulates RUNX2 directly or through regulation of the Wnt pathway antagonist *DKK1*, promoting osteogenic differentiation in early osteoblasts and mesenchymal stem cells (MSC). Adapted from data and figures from paper III and (Li et al. 2008; Scarola et al. 2010; Tome et al. 2010; Zhang et al. 2011a).

*Cell lines as model systems for cancer specific methylation patterns*

One major concern when using cell lines as model systems is how closely related they are to the primary tumours from which they originated, how accurately a particular cell type represents the general population of primary tumours and how *in vitro* conditions mimic those *in vivo*. Since epigenetic information *per se* is reversible information, it is particularly important to separate what underlies the transformed phenotype and what is only induced by cell culture. The methylation profiles of normal and tumour tissues of lung and colon, and permanent cancer cell lines originally derived from these tissues were compared in a study from Lewin et al. (Lewin et al. 2007). The cancer specific profiles were very similar for the two tumour types indicating that the tissue specific DNA methylation is largely lost in cultivated cancer cell lines. Moreover, the cell lines represented the DNA methylation pattern of the corresponding primary tissue rather well. However, cancer cell lines have in general demonstrated an increased frequency of CpG island hypermethylation compared to their primary counterpart (Smiraglia et al. 2001; Paz et al. 2003). In our study we observed that the cell lines were highly methylated with a consistency between methylation and expression level, while the osteosarcoma tumour samples generally showed a lower methylation

frequency or lower level of methylation, showing that care must be taken when using immortalized cell lines as model systems as a basis for the evaluation of cancer specific methylation patterns.

## CONCLUSIONS AND FUTURE PERSPECTIVES

*Conclusions and further perspectives for the present projects*

In this thesis, genome-wide patterns of gene expression at the miRNA and mRNA, level as well as DNA methylation in osteosarcoma have been studied.

In paper I, it was revealed that expression of *CXCR4* in primary osteosarcoma samples was associated with subsequent development of metastases, suggesting a prognostic value in osteosarcoma. These finding should be confirmed at the protein level in a larger panel using immunohistochemistry or tissue microarrays. The detail expression of CXCR4 in tumour, stroma or infiltrating cells should be investigated. Extended studies on an independent cohort of clinical samples may validate CXCR4 expression as a prognostic marker for metastasis in osteosarcomas.

In paper II, we identified miRNAs that were differently expressed between osteosarcomas and normal samples, suggesting a role in osteosarcoma biology. Our research group recently published an article on the integration of datasets of mRNA gene expression, copy number and DNA methylation in osteosarcoma cell lines (Kresse et al. 2012b). Similar datasets for gene expression (mRNA and miRNA), DNA methylation and copy number are available for xenografts and osteosarcoma clinical samples. These datasets will be integrated in order to identify genes that may play an important role in osteosarcoma development. These studies will also be used to validate the importance of an integrated approach to identify possible driver genes in osteosarcomas, as well as validate our initial finding s using cell line models. DNA methylation data for the 450k array generated in paper III will be used for verification of epigenetic changes in patients.

In paper III, we identified miRNA loci that were highly methylated in osteosarcoma compared to normal samples. From the present findings we conclude that *mir-335* and *mir-*486 is methylated in osteosarcoma cell lines and clinical samples. Methylation of *mir-335* and

*mir-485* was also detected in clincal samples, and miR-34c, miR-486-5p and miR-335 show a tumour suppressive role in osteosarcoma. Additional experiments are warranted in this project. The distal CGI of miR-335 has to be examined with qMSP in patient samples. The upstream island of miR-486 should be characterized using bisulfite sequencing, providing a higher resolution at single CpG level in order to verify the results of the qMSP data for a selected group of tumour samples. In addition, the relation between DNA methylation and miRNA expression has to be confirmed in patient tumour samples.

*From microarray profiling to high-throughput deep sequencing*

In the early studies of miRNAs, custom home-made arrays were extensively used, followed by commercial miRNA microarrays. When our studies on miRNAs in osteosarcoma were initiated, 799 miRNAs could be profiled using one of the most comprehensive commercial miRNA microarrays available, the Agilent human miRNA microarrays version 2, based on the Sanger miRBase database release 10.1 (www.mirbase.org). The most recent version of Agilent's miRNA microarrays is based on miRBase 18 containing 1,921 mature miRNAs. The number of identified miRNAs continues to increase, and in the last update of miRBase (version 19) 1,600 precursor miRNAs and 2,042 mature human miRNAs were registered. However, miRNA microarrays are restricted to detection and profiling of previously identified miRNA sequences, and despite this rapid growth, many miRNAs have not yet been validated, and several others are yet to be identified.

The last generation of large-scale profiling methods is represented by the high-throughput sequencing, which relies on next generation sequencers. High-throughput or deep sequencing not only provides quantification of expression, but also characterizes miRNA abundance and nucleotide variation and allow for identification of unknown miRNAs, offering a more accurate map of all expressed small RNAs genome-wide. These methods are exceptionally useful as they may accommodate a large number of samples in a cost-effective manner, albeit deep sequencing produces large datasets and presents formidable computational challenges. Recently, small RNA deep sequencing, among other studies, been used to perform miRNA sequence and expression analysis in breast cancer (Farazi et al. 2011), to reveal the deregulation of the miRNA biogenesis pathway in human glioma (Moore et al. 2012) and

reported the importance of isomiR variants (Neilsen et al. 2012). As for today, efforts on studying small RNAs will increasingly be based on deep sequencing methods identifying a large number of new small RNAs and a dramatic increase in the sensitivity of detection.

Several methods have been developed to map DNA methylation on a genomic scale. The probes of the Infinium assay cover only a small percentage of all CpGs in the genome and are preferentially located in unmethylated promoter regions. The availability of reference genome assemblies and deep sequencing has led to methods that provide high-resolution, genome-wide profiles of CpGs. In contrast to arrays, sequencing-based methods can identify DNA methylation genome-wide at a single nucleotide level, including repetitive sequences and also in non-regulatory regions. In addition, single nucleotide sequencing allows the determination of epigenetic states at allele-specific level (Bock et al. 2010; Harris et al. 2010). These new technologies are rapidly becoming affordable and accessible, and soon will be used in many epigenetic studies.

*Towards personalized cancer treatment*

As deep sequencing technologies are now rapidly becoming available at a reduced cost, a detailed molecular characterization of all DNA alterations of each tumour is becoming feasible. The vision of the war on cancer has transformed from the seeking of a specific cure for each cancer type to finding a specific cure for each cancer patient, moving towards a personalized cancer treatment. Mutations in the same genetic pathways may occur in many different tumour types. By using mutation information, oncologist in a near future may be able to choose the correct drugs that targets specific altered pathways. Thus, next-generation sequencing technologies can hopefully be used to establish the appropriate drug treatment for each individual patient. As part of the Norwegian Cancer Genome Consortium we are involved in a national personal cancer medicine project. This project will establish standardised routines and provide methodology and bioinformatics tools for deep sequencing, identify mutations in thousands of patient samples by comparing sequences from tumours with matched patient blood samples, establish nation-wide cancer databases of known targetable genes and evaluate the use for guidance of targeted therapies across cancer types.

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*Research Article*

# Global Gene Expression Profiling of Human Osteosarcomas Reveals Metastasis-Associated Chemokine Pattern

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Global gene expression analysis was performed on a panel of 23 osteosarcoma samples of primary and metastatic origin using the Applied Biosystems Gene Expression Array System. When comparing the primary tumours with the metastases, we found a significantly increased expression of genes involved in immunological processes, for example coding for cytokines and chemokines, in the metastatic samples. In addition, a comparison of the gene expression in primary samples from patients with or without metastases demonstrated that patients who later developed metastases had high expression of the chemokine (C-X-C motif) receptor 4 (*CXCR4*), similar to the metastatic samples, suggesting that these signal molecules play an important role in promoting metastasis. Increased knowledge of mechanisms and interactions between specified molecular signalling pathways in osteosarcomas could lead to a more rational strategy for development of targeted therapy.

## 1. Introduction

Osteosarcoma is the most frequent primary malignant tumour of bone in children and adolescents, with a peak incidence at the age of 15–19 years, and has an annual incidence of 4/million/year world-wide (reviewed in [1, 2]). Osteosarcomas are rare, osteoid-producing malignant tumours that usually arise in the metaphyseal regions of long bones, in particular, the distal femur, proximal tibia, and proximal humerus [2]. Although most osteosarcomas are diagnosed without a predisposing condition, approximately 15% arise in adults secondary to a predisposing genetic condition (Li-Fraumeni syndrome, hereditary retinoblastoma; RB), disease (Paget disease of bone), or prior treatment (radiation) [2].

Most conventional osteosarcomas are high-grade tumours with a complex karyotype that displays numerous genetic aberrations [2]. Despite the advances in multimodal treatment combining adjuvant chemotherapy and surgical-wide resection, the 5-year survival rate for patients diagnosed with osteosarcoma without presence of metastasis remains in the order of 60–65% (reviewed in [3, 4]). Metastases are the leading cause of cancer-related death, and around 13–27% of the osteosarcoma patients have detectable metastases at diagnosis [3–6], whereas 40% will develop metastases at a later stage [4]. The metastatic process shows a tropism for lungs (80%), with skeleton as the second most common site [4]. The 5-year survival rate for osteosarcoma patients with primary metastases is in the range of 20–29% [4, 7, 8].

Molecular pathways contributing to osteosarcoma development and progression have recently been discovered (reviewed in [9, 10]), and this may facilitate better diagnosis and prognostication, as well as the development of new treatment strategies. The molecular alterations contributing to metastasis in osteosarcomas are increasingly being understood (reviewed in [4]), and several studies have employed microarray gene expression profiling to identify genes involved in the metastasis process. Comparisons of high- and low-metastatic osteosarcoma cell lines using microarrays have identified several differentially expressed genes related to growth arrest and apoptosis [11], as well as adherence, motility, and/or invasiveness [12]. Another study identified two genes not previously linked to osteosarcoma, epiregulin (*EREG*) and carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2 (*CHST2*), both predictive for survival [13]. A recent gene expression profiling of osteosarcoma patients who did and did not develop metastasis revealed a number of differentially expressed genes related to immunological functions, particularly macrophages [14].

Due to the high incidence of metastasis and low survival rate in metastatic osteosarcoma patients, we wanted to investigate the differences in gene expression pattern between primary and metastatic tumours. Microarray gene expression analysis was performed on a panel of 23 osteosarcoma samples of primary and metastatic origin, and the expression patterns were compared in order to identify differentially expressed genes and molecular signalling pathways involved.

## 2. Materials and Methods

*2.1. Biological Material.* Twenty-three human osteosarcomas were selected from a tumour panel at the Department of Tumour Biology at the Norwegian Radium Hospital, collected during two decades (1983–2004). Clinical samples were collected immediately after surgery, cut into small pieces, frozen in liquid nitrogen, and stored at $-80^{\circ}$C until use. The informed consent used and the collection of samples were approved by the Ethical Committee of Southern Norway (Project S-06133).

All tumours were revised at the time of the study by the pathologist (BB) and diagnosed according to the current World Health Organization classification [2]. The clinical information was retrieved anonymously from the MEDinsight database at the Norwegian Radium Hospital (http://medinfo.net/medinsight/). The panel consisted of 12 primary tumours obtained from open biopsies or surgical specimens and 11 tumours obtained from surgical specimens of distant metastases. Of the 12 primary tumours, 7 were from patients who later developed metastases and 4 from those who did not (clinical information about the metastatic process was not available for one primary tumour). The follow-up of the patients who did not develop metastasis was in the range 5–19 years. Median patient age was 18 (range 11–50), and the gender ratio (female : male) was 1 : 1.3. Clinical information on the tumour samples are given in Table 1.

Two normal bone samples were included for the quantitative real-time RT-PCR experiments. Bone1 was collected from the femur of a renal cell carcinoma patient and Bone2 from the tibia of an osteosarcoma patient. For both patients, the normal samples were collected distant from the margin of the tumours.

*2.2. Microarray Experiments.* The microarray experiments were performed with the Applied Biosystems Gene Expression Array System, and the samples were hybridised on the Human Genome Survey Microarray V2.0 (Applied Biosystems, Foster City, CA, USA) with 32 k probes covering 29 k genes. The complete dataset can be viewed in the Gene Expression Omnibus (GEO) microarray database (http://www.ncbi.nlm.nih.gov/geo/, accession number GSE32981).

*2.3. RNA Isolation and Hybridization.* RNA was extracted by TRIzol (OS3, 4, 12, 18, 21, 41, 47, 48, 50, 51, 53, 55) or GTC (OS11, 14, 15, 17, 19, 20, 23, 25, 29, 30, and 32) using standard protocols. RNA purity and quantity were measured on a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), and RNA integrity was evaluated on an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA) using the total-RNA chip. The RNA was amplified, hybridised on the Human Genome Survey Microarray V2.0 (Applied Biosystems), and scanned according to the manufacturer's protocols.

*2.4. Preprocessing and Filtering.* The microarray image files were pre-processed with the AB1700 software (Applied Biosystems) and the resulting data files were stored in BASE [15]. The data were further processed with the R package ABarray, which is part of the Bioconductor project (http://www.R-project.org/) [16]. The data were quantile normalized, $\log_2$ transformed, and missing values were imputed using average values from the other arrays in the subgroup. The osteosarcoma samples were divided into two groups of primary or metastatic origin. Weakly expressed probes were filtered away by defining that a probe is only detected if it has a signal-to-noise ratio (SNR) $\geq 3$ in at least 50% of the samples in either subgroup primary or metastatic.

*2.5. Data Analysis.* Using the database Panther [17, 18], a Wilcoxon Rank-Sum test was performed to identify enriched biological pathways using all of the genes that were expressed above the detection limit and mapped to Celera ID.

The data were imported to J-Express 2.7 [19], and the values were merged to gene level using the max probe function. The Statistical Analysis of Microarrays (SAM) plug-in [20] was used to identify differentially expressed genes between the primary and the metastatic samples, as well as between the primary samples that did and did not develop into metastases. Significant genes were identified using the $d$-value, which measures the strength of the relationship between gene expression and the response variable. An analysis was performed on the discriminatory gene lists where the genes were categorized based on enriched

Table 1: Clinical information on the osteosarcoma samples.

| Sample | Sample origin | Patient age/sex | Subtype | Grade[1] | Primary location | Metastasis location | Chemotherapy[2] | Response[3] | Treated sample | Metastasis (months)[4] | Follow-up (months)[5] | Status |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OS3 | Met | 17/M | Obl/Cho | 4 | Femur | Lung | Yes (SSG II + VI) | NA | Yes | 23 | 45 | DD |
| OS4 | Met | 15/M | Obl | 4 | Femur | Lung | Yes (SSG II) | Poor | Yes | 14 | 37 | DD |
| OS11 | Met | 22/M | Par | 4 | Femur | Multiple[6] | No | — | No | 185 | 193 | DD |
| OS12 | Met | 41/M | Obl/Cho | 4 | Femur | Lung | Yes (SSG VI + VIII) | NA | Yes | 9 | 49 | DD |
| OS17 | Met | 18/M | Obl + Cho | 4 | Humerus | Lung | Yes (SSG II + VI) | NA | Yes | 25 | 49 | DD |
| OS18 | Met | 26/M | Obl | 3 | Fibula | Lung | No | — | No | 117 | 353 | DD |
| OS21 | Met | 17/F | Obl | 4 | Femur | Lung | Yes (SSG VIII) | Poor | Yes | MD | 33 | DD |
| OS23 | Met | 11/F | Obl | 4 | Femur | Lung | Yes (SSG VIII) | NA | Yes | MD | 229 | NED |
| OS25 | Met | 19/M | Fbl | 4 | Fibula | Skeleton | Yes (SSG VI + VIII) | NA | Yes | MD | 44 | DD |
| OS32 | Met | 24/M | Fbl | 4 | Tibia | Lung | Yes (SSG VI) | Poor | Yes | 6 | 208 | NED |
| OS53 | Met | 18/M | Obl/Fbl | 4 | Humerus | Multiple[7] | Yes (ISG/SSG I) | Poor | Yes | 26 | 68 | DD |
| OS14 | Prim | 14/F | Obl/Fbl | 4 | Femur | Lung | Yes (SSG II) | Poor | Yes | MD | 13 | DD |
| OS15 | Prim | 49/F | Obl | 4 | Costa | Lung | Yes (NA) | NA | No | MD | 3 | DD |
| OS19 | Prim | 16/F | Obl | 4 | Tibia | — | Yes (SSG VIII) | NA | Yes | — | 245 | NED |
| OS20 | Prim | 17/F | Obl/Fbl | 4 | Humerus | Soft tissue | Yes (SSG VIII) | NA | No | 139 | 151 | DD |
| OS29 | Prim | 27/F | Obl | 4 | Pelvis | Lung | No | — | No | MD | 4 | DD |
| OS30 | Prim | 21/F | Obl | 4 | Femur | Lung | Yes (SSG VIII) | NA | No | MD | 162 | NED |
| OS41 | Prim | 11/M | Obl | 4 | Tibia | NA | No | — | No | NA | NA | NA |
| OS47 | Prim | 12/M | SC/Pleo | 4 | Humerus | Lung | Yes (SSG XIV + EURAMOS I) | Good | No | 92 | 124 | NED |
| OS48 | Prim | 50/M | Obl/Fbl | 4 | Tibia | — | Yes (SSG XIV) | NA | No | — | 74 | NED |
| OS50 | Prim | 11/M | Cho | 4 | Femur | — | Yes (SSG XIV) | Poor | No | — | 108 | NED |
| OS51 | Prim | 18/F | Obl/Tel | 4 | Femur | Lung | Yes (ISG/SSG II) | NA | No | MD | 22 | DD |
| OS55 | Prim | 17/F | Obl | 4 | Femur | — | Yes (SSG XIV) | NA | No | — | 91 | NED |

Abbreviations: OS, osteosarcoma; Met, metastasis; Prim, primary tumour; M, male; F, female; Obl, osteoblastic; Cho, chondroblastic; Par, parosteal; Fbl, fibroblastic; SC, spindle cell; Pleo, pleomorphic; Tel, telangiectatic; NA, not available; DD, dead of disease; NED, no evidence of disease.

[1] Grading is based on a four-tiered system used in the Scandinavian Sarcoma Group (SSG).

[2] Chemotherapy has been given according to the indicated Scandinavian Sarcoma Group (SSG) protocols, Italian Sarcoma Group/Scandinavian Sarcoma Group (ISG/SSG) protocol or European, and American Osteosarcoma Study Group (EURAMOS) protocol (for more information, see http://www.ssg-org.net/index.htm).

[3] Histological evaluation.

[4] Time to first metastasis from diagnosis.

[5] Time to last follow-up from diagnosis.

[6] Multiple locations, lung, and lymph node.

[7] Multiple locations, lung, and skeleton.

Table 2: Enriched Panther pathways in the metastases compared to the primary tumours. The five most significant pathways are shown with the number of genes in the pathways. + or − signs indicate that for the genes belonging to this pathway, the distribution of fold change values is shifted towards higher or lower values, respectively, than the overall distribution of all genes that were uploaded. *P*-values were calculated from the Wilcoxon Rank-Sum test and Bonferroni corrected for multiple testing.

| Pathways | # Genes | +/− | *P*-value |
|---|---|---|---|
| T cell activation | 134 | + | 5.5E–14 |
| Inflammation mediated by chemokine and cytokine signaling pathway | 299 | + | 2.1E–11 |
| B cell activation | 92 | + | 7.2E–7 |
| EGF receptor signaling pathway | 175 | + | 1.0E–3 |
| Integrin signaling pathway | 244 | + | 1.8E–3 |

Gene Ontology (GO) processes using Panther. Hierarchical clustering was done in J-Express, and the gene expression is given relative to the mean expression level in all the samples.

*2.6. Quantitative Real-Time RT-PCR.* Quantitative real-time RT-PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems, California, USA). The expression level of the gene chemokine (C-X-C motif) receptor 4 (*CXCR4*, assay ID Hs00237052_m1) was determined in 19 of the tumour samples and 2 normal bone samples. The genes beta-2-microglobulin (*B2M*, assay ID Hs99999907_m1), TATA-box binding protein (*TBP*, assay ID Hs99999910_m1), and eukaryotic 18S rRNA (*18S*, assay ID Hs99999901_s1) were used as endogenous controls for normalization. Universal Human Reference RNA (Stratagene, California, USA) was used as a reference.

The High Capacity cDNA Archive Kit (Life Technologies) was used to synthesize cDNA, and real-time PCR was performed using the ABI 7500 Real Time PCR System and software, essentially as described in the protocol supplied by the manufacturer (Applied Biosystems). Each assay included (in duplicate) a standard curve of six serial dilutions of the Universal Human Reference RNA cDNA (ranging from 100 ng to 100 pg), 10 ng of each tumour and normal bone cDNA, and a no-template control. The expression levels were determined from the standard curves as described by the manufacturer. The expression level of *CXCR4* was normalized with the average expression of the three endogenous controls, and the relative expression of the tumour samples was compared to the average expression of the two normal bone samples.

## 3. Results

*3.1. Comparison of Primary and Metastatic Tumours.* Gene expression profiling was performed on a panel of 23 human osteosarcoma samples of primary and metastatic origin. The clinical information on the tumour samples is given in Table 1. Twelve primary and 11 metastatic samples were analysed to identify differences in gene expression and pathways with enrichment of differentially expressed genes between the two groups. After SNR filtering, 22,510 genes were expressed above the detection limit, of which 21,378 had Celera IDs. Unsupervised hierarchical clustering of all the samples revealed no specific patterns, and the primary and metastatic samples clustered intermingled (data not shown). The analysis using Panther identified several pathways with enriched differential expression between the primary and metastatic samples, and these are listed in Table 2. The genes in the pathway were generally higher expressed in the metastases than in the primary samples. The most prominent pathways are involved in immunological processes and chemokine and cytokine signalling, as well as pathways like the EGF receptor signalling pathway, including genes coding for the FBJ murine osteosarcoma viral oncogene homolog (*FOS*), early growth response 1 and -2 (*EGR1* and *-2*).

The primary and metastatic samples were further compared by SAM analysis in order to identify the most differentially expressed genes separating these two groups, and the top-210 gene list based on the *d*-value is given in Supplementary Table 1 available at doi:10.1155/2012/639038. The majority of these genes were upregulated in the metastases compared to the primary tumours. Figure 1 shows the hierarchical clustering of all the tumours based on the expression pattern of these 210 genes (the same figure with the gene names indicated is given in Supplementary Figure 1). The samples were separated into two main clusters, one consisting of all primary and a subset of the metastatic samples and one with only metastatic samples. Among the primary samples, there was one subcluster that strongly diverged from the other samples, consisting of three samples that did not metastasize and the one sample with unknown metastatic status. Among the genes separating the metastatic samples into different subclusters, a high number of surfactants were present, being highly expressed in all the samples of the subcluster that only contained lung metastases. In addition, several interesting clusters of genes were observed to be upregulated in both of the metastatic subclusters, like the group consisting of chemokine (C-X-C motif) ligand 1, -2, and -3 (*CXCL1*, *-2*, and *-3*) and interleukin 6 (interferon, beta 2) (*IL6*), as well as the group consisting of *EGR1*, *-2*, *FOSB*, *FOS*, and jun B proto-oncogene (*JUNB*), both highlighted (blue and green colour, resp.) in Figure 1.

To further explore the function of these 210 differentially expressed genes, they were classified into Gene Ontology (GO) Biological process and Molecular function, listed in Table 3. Interestingly, there was a significant overrepresentation of genes involved in immunity and defence, especially cytokines and chemokines like *CXCL1*, *-2*, *-3*, *-5*, *IL1B*, *-6*, *-8*, *-17D* and oncostatin M (*OSM*). These signal molecules have a higher expression in metastatic samples compared

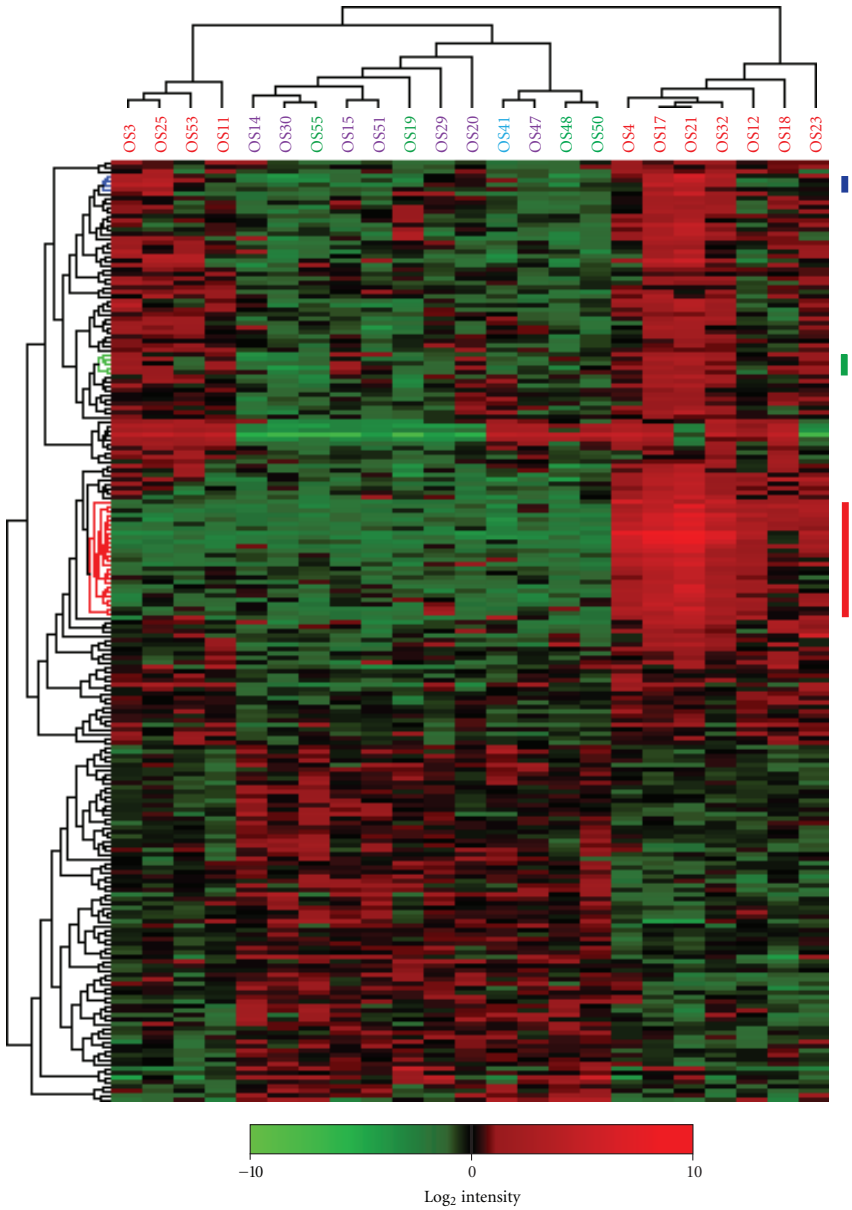


Figure 1: Hierarchical clustering of all tumours based on the top-210 significant genes differentially expressed between metastases and primary tumours, identified by SAM analysis. Samples coloured in red, metastases; lilac, primary samples from patients who later developed metastases; green, primary samples from patients who did not develop metastases blue, clinical information on metastases not available. Gene cluster coloured in blue, *CXCL1, -2, -3, IL6,* and *LOC131873*; green, *EGR1, -2, FOSB, FOS,* and *JUNB*; red, surfactant genes. Red, increased gene expression; green, decreased gene expression. The cluster was made using average linkage and Pearson's correlation.

Table 3: GO groups with significant enrichment, based on a comparison of the top-210 significant genes differentially expressed between metastases and primary tumours against all genes present on the microarray. The number of genes in the total list and observed and expected number of genes in the gene list that map to the GO group are shown. + or − signs indicate over- or underrepresentation, respectively, of this GO group. *P*-values were Bonferroni corrected for multiple testing, *P*-value < 0.05.

| GO group | # Genes total | Gene list | | | |
|---|---|---|---|---|---|
| | | # Observed | # Expected | +/− | *P*-value |
| *Biological process* | | | | | |
| Immunity and defence | 1365 | 30 | 11.3 | + | 3.7E–5 |
| Granulocyte mediated immunity | 59 | 6 | 0.5 | + | 1.7E–3 |
| Macrophage mediated immunity | 126 | 8 | 1.1 | + | 1.8E–3 |
| Cell proliferation and differentiation | 944 | 20 | 7.8 | + | 4.2E–3 |
| Blood circulation and gas exchange | 82 | 5 | 0.7 | + | 2.0E–2 |
| Cell cycle control | 390 | 12 | 3.2 | + | 1.7E–2 |
| JNK cascade | 60 | 5 | 0.5 | + | 3.2E–2 |
| *Molecular function* | | | | | |
| Surfactant | 9 | 5 | 0.7 | + | 2.8E–6 |
| Chemokine | 45 | 5 | 0.4 | + | 6.8E–3 |
| Interleukin | 34 | 4 | 0.3 | + | 4.1E–2 |

with primary samples, and the high expression level seems to be strongly associated with metastasis. In addition, a group of surfactants, surfactant protein A1, -A2, -B, -C, and -D (*SFTPA1*, *-A2*, *-B*, *-C,* and *-D*) was observed to be higher expressed in a subset of the metastatic samples, contributing to a gene cluster that distinguished between lung and non-lung metastases (coloured red in Figure 1).

*3.2. Comparison of Primary Tumours with Different Capability to Metastasize.* To further look into the genes apparently involved in the metastatic process, the primary samples from the patients who developed metastases were compared with those who did not. A SAM analysis of seven primary samples that metastasized and four that did not resulted in a short list of genes that were differentially expressed between the two groups of primary samples. The top-20 gene list based on the *d*-value is shown in Table 4. Figure 2 shows the hierarchical clustering of the primary samples based on the expression of these 20 genes. The primary samples that developed into metastases clustered separately from the primary samples that did not. Thirteen of the differentially expressed genes were upregulated in the primary samples that developed into metastases, whereas seven genes were downregulated. Among the genes that were upregulated in the primary samples from patients who later developed metastases was the chemokine (C-X-C motif) receptor 4 (*CXCR4*), known to be involved in the metastatic process.

Figure 3(a) shows the expression level of the *CXCR4* gene in all the samples. *CXCR4* was expressed at the same level in metastatic samples and in primary samples that developed metastases, with a significantly lower expression level in primary samples that did not metastasize compared to the two other groups ($P < 0.001$ for both comparisons). The expression was also significantly different between all the primary samples combined and the metastatic samples ($P < 0.05$). The expression level was also determined in 19 of the tumour samples compared to two normal bone samples using quantitative real-time RT-PCR, and this is shown in Figure 3(b). The relative expression levels were in general similar using the two methods, with higher expression of *CXCR4* in the primary samples that developed metastases and the metastatic samples. Most samples showed similar expression levels, only OS14 was markedly different. However, there were smaller relative differences in expression level between the samples based on the quantitative real-time RT-PCR data, and the expression was not significantly different between the groups here.

## 4. Discussion

Osteosarcomas show complex genomic changes with few, if any, consistent chromosomal aberrations, which makes it difficult to identify the molecular features that underlie the development of this type of cancer. The aim of this study was to investigate the differences in gene expression pattern between primary and metastatic tumours, and a number of pathways and genes with differential expression were identified (Tables 2 and 3 and Figure 1). In general, the genes were more highly expressed in the metastases than in the primary osteosarcoma tumour samples. Of particular interest, we identified a number of immunological processes, including the T cell and B cell activation, as well as chemokine and cytokine signalling pathways. Chemokines are chemotactic cytokines, a family of small cytokines or proteins secreted by cells, and the major role of chemokines is to control and direct the migration of cells. It has been increasingly known that chemokines play an important part in regulation of the metastatic cascade in a wide range of tumours, including osteosarcomas [21–24]. Tumour cells that are attracted by chemokines have an increased expression of particular chemokine receptors on their surface. The cells migrate towards a signal of increasing chemokine

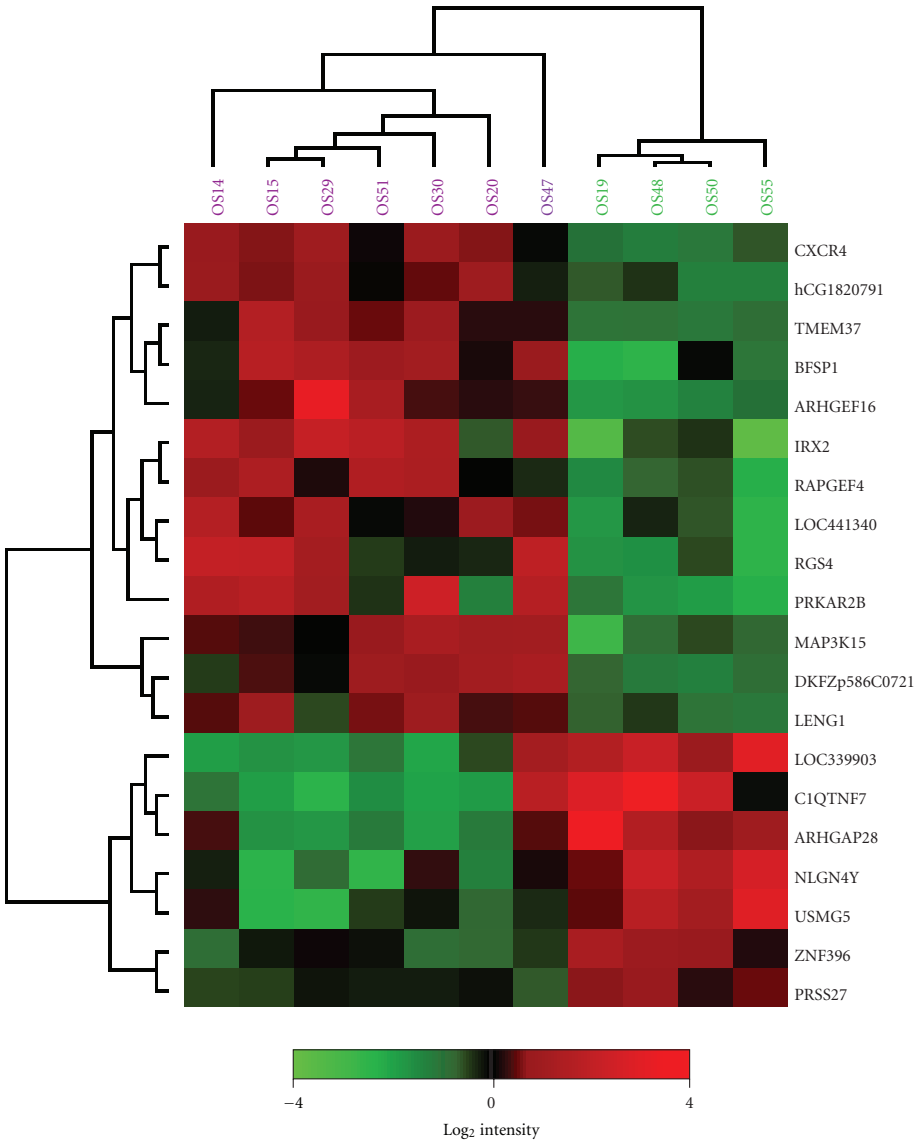


Figure 2: Hierarchical clustering of all primary tumours based on the top-20 significant genes differentially expressed between primary samples from patients who developed metastases and those who did not, identified by SAM analysis (one primary tumour with no clinical information on metastases was excluded). Samples coloured in lilac, primary samples from patients who later developed metastases; green, primary samples from patients who did not develop metastases. Red, increased gene expression; green, decreased gene expression. The cluster was made using average linkage and Pearson's correlation.

concentration provided by the source of the chemokine, and this process enables them to migrate to secondary tissues where chemokine ligands are highly expressed. Part of the identified differences between primary and metastatic samples could be due to the origin of the samples, although the histology of primary and pulmonary metastatic osteosarcoma samples has been reported to be similar in about 60% of the cases [25, 26].

The chemokines *CXCL1*, *-2*, *-3*, *-5*, and *IL8* (*CXCL8*) were among the top-210 genes that were observed to be

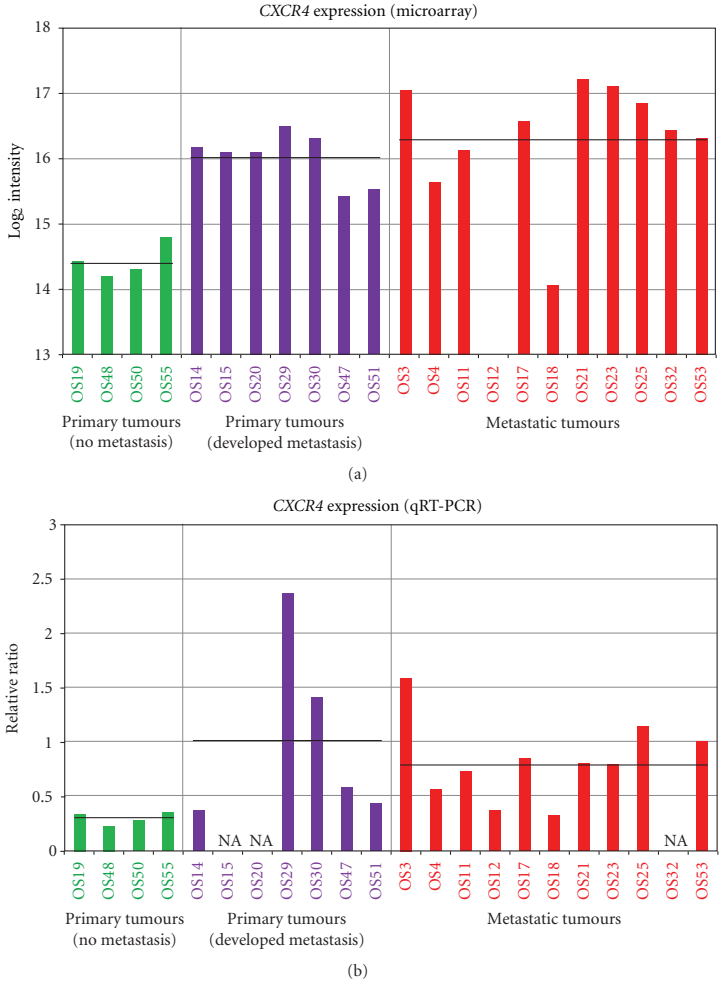


Figure 3: Relative *CXCR4* expression in primary tumours from patients who did or did not develop metastases and in metastatic samples (one primary tumour with no clinical information on metastases was excluded) based on (a) microarray data and (b) quantitative real-time RT-PCR data. For the quantitative real-time RT-PCR data, the expression levels have been normalised to the average expression of three housekeeping genes (*18S*, *B2M* and *TBP*) and compared to the average expression of two normal bone samples. The average expression level in each sample group is indicated with a black bar. NA, not available.

upregulated in metastases compared with primary tumours, and they showed highly correlated expression patterns (Figure 1, Supplementary Table 1, and Supplementary Figure 1). This is similar to previous observations in breast cancer tumours and cell lines, where these chemokines have been suggested to account for a higher aggressiveness of ER$\alpha$-positive tumours [27]. These genes are located in the 4q21 region, and although the gene cluster was not amplified in breast cancer, it was observed to be coregulated at the transcriptional level. Regions in 4q have been shown to have increased copy number in osteosarcomas [28, 29], but amplification of the 4q21 region has not recurrently been reported. In breast cancer, *CXCL8* expression level strongly correlated with activating transcription factor 3 (*ATF3*), c-Jun and JunB, members of the AP-1 transcription factor complex [27], and *ATF3* and *JunB* were also observed to be upregulated in the metastatic osteosarcoma samples investigated here.

Several other chemokines were upregulated in the metastatic osteosarcomas, including *OSM*, which has been claimed to have both pro-inflammatory and anti-inflammatory effects. OSM has been shown to induce bone loss and

Table 4: Top-20 genes identified by SAM analysis as differentially expressed in the comparison of primary samples from patients who developed metastases and those who did not.

| Gene symbol | Gene name | Fold change | *d*-value |
|---|---|---|---|
| CXCR4 | Chemokine (C-X-C motif) receptor 4 | 3.1 | 4.3 |
| LOC339903 | — | −8.6 | −3.7 |
| TMEM37 | Transmembrane protein 37 | 3.1 | 3.6 |
| MAP3K15 | Mitogen-activated protein kinase kinase kinase 15 | 4.1 | 3.4 |
| BFSP1 | Beaded filament structural protein 1, filensin | 5.1 | 3.4 |
| DKFZp586C0721 | — | 3.2 | 3.4 |
| hCG1820791 | — | 2.7 | 3.3 |
| IRX2 | Iroquois homeobox protein 2 | 9.2 | 3.3 |
| NLGN4Y | Neuroligin 4, Y-linked | −6.9 | −3.3 |
| C1QTNF7 | C1q and tumor necrosis factor related protein 7 | −12.5 | −3.2 |
| ARHGAP28 | Rho GTPase activating protein 28 | −6.7 | −3.2 |
| PRKAR2B | Protein kinase, cAMP-dependent, regulatory, type II, beta | 6.8 | 3.2 |
| RAPGEF4 | Rap guanine nucleotide exchange factor (GEF) 4 | 4.1 | 3.2 |
| LOC441340 | — | 4.1 | 3.2 |
| RGS4 | Regulator of G-protein signalling 4 | 5.8 | 3.1 |
| ZNF396 | Zinc finger protein 396 | −2.4 | −3.1 |
| PRSS27 | Protease, serine 27 | −1.8 | −3.1 |
| USMG5 | Upregulated during skeletal muscle growth 5 | −5.9 | −3.0 |
| LENG1 | Leukocyte receptor cluster (LRC) member 1 | 2.5 | 3.0 |
| ARHGEF16 | Rho guanine exchange factor (GEF) 16 | 4.9 | 3.0 |

sensitized rat osteosarcoma to apoptosis [30] and induce differentiation of chondrosarcoma and osteosarcoma cells [31, 32]. However, several studies show that OSM may enhance tumour progression and metastasis. Stimulation of human and canine osteosarcoma cells by OSM has been shown to promote invasive behaviour through activation of signal transducer and activator of transcription 3 (acute-phase response factor, STAT3) [33], and OSM treatment increased migration and enhanced epithelial-to-mesenchymal transition in several tumours [34–36].

The hierarchical clustering based on the top-210 differentially expressed genes separated the metastatic samples into two subclusters (Figure 1). The smaller metastatic cluster, which was more similar to the primary samples, consisted of one pulmonary, one skeletal, and two samples that formed multiple metastases of both the lungs and skeleton. The major cluster of metastatic samples only included pulmonary metastases, and these samples showed high expression of surfactants. SP-A, SP-B, SP-C, and SP-D are expressed by type II alveolar epithelia or Clara cells, assumed to be progenitors of pulmonary adenocarcinomas, and have been used as markers of metastatic and micrometastatic pulmonary adenocarcinoma and nonsmall cell lung carcinomas [37]. It seems likely that the detection of these surfactants may be due to contamination of lung tissue in the tumour samples, although it is also possible that the lung microenvironment induces these genes in the osteosarcoma cancer cells.

Interestingly, in the comparison of primary tumours with different capability to metastasize, primary samples that did not metastasize showed significantly lower expression of the chemokine receptor *CXCR4* than primary samples from patients who later developed metastases ($P < 0.001$) (Table 4 and Figures 2 and 3(a)). The expression level was similar between the primary samples that metastasized and the metastatic samples. Although *CXCR4* was not among the top-ranked genes from the SAM analysis of primary tumours and metastases, the expression level was significantly different between all the primary tumours and the metastases ($P < 0.05$), most likely due to the lower level of expression in the primary samples that did not metastasize. The expression level was also confirmed using quantitative real-time RT-PCR (Figure 3(b)), and although the patterns in general were similar between the two methods, no significant differences in expression were observed between the groups based on this data.

Two of the metastatic samples, OS11 and OS18, originated from patients that did not develop metastases until 15 and 10 years after diagnosis, respectively. OS11 is a parosteal osteosarcoma but expressed *CXCR4* at the same level as the other metastatic samples, while OS18 showed a low level of *CXCR4* expression. Both samples were untreated, in contrast to the other metastatic samples, but the clustering analyses showed that these two cases were not different from the other metastatic samples, justifying the inclusion of these samples in the study. Similarly, two primary samples were treated (OS14 and OS19), in contrast to the other primary samples, but these were also not different from the other primary samples. However, OS14 was the only samples showing markedly different expression level of *CXCR4* depending on the method used.

This study is the first to use expression profiling to identify that differential expression of *CXCR4* separates

primary samples with different capabilities to metastasize. Although *CXCR4* was not among the identified genes in a recent publication by Buddingh et al. [14], the significance of CXCR4 in metastasis development in osteosarcoma and other bone cancers has previously been reported. CXCR4 is a specific receptor for the ligand SDF-1 (stromal-derived-factor-1, also called CXCL12), and the CXCR4/CXCL12 axis has been shown to be important for tumour progression in a high number of cancer types [38]. In a mouse model, the tumour cells with CXCR4 receptor were chemoattracted by CXCL12, migrated through the lymphatic and vascular system, and arrested in CXCL12 rich organs like the bone and lungs [39]. Laverdiere et al. [24] observed no significant difference between *CXCR4* expression in primary and metastatic samples, but the level of *CXCR4* expression was inversely correlated with the presence of metastases at diagnosis and survival (event-free survival and metastatic-free survival), as patients with tumors expressing *CXCR4* had a worse survival. However, in a study by Oda et al. [40], higher immunohistochemical CXCR4 expression was observed in metastases compared with primary tumours. Contradicting the above observation, a higher frequency of canine osteosarcoma primary tumours than pulmonary metastases expressed CXCR4 protein [41]. In an analysis of Ewing sarcoma, another bone cancer, *CXCR4* correlated with metastases, and *CXCR4* in combination with *CXCR7* were shown to be prognostic indicators for patient survival [23]. In chondrosarcoma of bone, CXCR4 showed higher immunohistochemical staining in high-grade than in low-grade samples, being a potential marker of aggressiveness [42].

A note to make is that the RNA samples used in this study were isolated using two different methods, which may influence the gene expression detected. The type of RNA isolation was randomly distributed between the groups of primary and metastatic samples, and with regard to other clinical properties (Table 1). Although it cannot be ruled out that the RNA isolation method has had an effect on the detected expression levels of *CXCR4*, the increasing evidence of a role of CXCR4 in osteosarcoma metastasis makes us believe that the expression differences observed here is due to the sample types and not the RNA isolation methods.

Osteosarcoma consists of both tumour cells derived from the mesenchyme as well as infiltrating mononuclear cells [43], hence chemokines could be expressed both by tumour cells as well as by the stroma cells in their proximity and at metastatic sites. Preliminary microarray results showed that *CXCR4* and interesting chemokine ligands like *CXCL1*, *-2*, *-3,* and *-5* are only highly expressed in clinical samples and not in xenografts or cell lines (Namløs et al., unpublished data), a finding supported by a previous study of osteosarcoma patient samples and cell lines [24]. This suggests that it is infiltrating stroma (macrophages) and not the tumour cells that is the major source of chemokine expression in osteosarcoma, or possibly that only the human macrophages are able to induce the expression of chemokines in the tumour cells. However, Perissinotto et al. [39] detected the CXCR4 receptor and a functional CXCR4/CXCL12 axis in non-confluent osteosarcoma cell lines. To further investigate this, immunohistochemical staining should be performed to examine the CXCR4 protein expression in the different cell types, which has previously been performed for other chemokine receptors in osteosarcoma [44].

Inhibition of the CXCR4/CXCL12 pathway by a peptide CXCR4 antagonist reduced the development of osteosarcoma murine lung metastases [45]. *In vivo* studies on breast cancer demonstrated that treatment with miRNA or antibodies against CXCR4 impaired migration and the development of murine lung metastases [21, 46]. This indicates that small molecule antagonists against CXCR4 can interfere with tumour progression and metastasis and may potentially be used for new therapeutic inventions.

## 5. Conclusion

Although the number of samples analysed was moderate, our results add to the increasing number of studies linking CXCR4 to metastasis, suggesting that the expression level of *CXCR4* may possibly be used as a prognostic factor in osteosarcoma. The identification of chemokine pathways that may promote cancer spread could give clinically useful biomarkers for the prediction of particularly aggressive tumours and might suggest therapeutic regimens that may target such tumours.

## Authors' Contributions

S. H. Kresse and C. R. Müller have equally contributed to this work.

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# Modulation of the Osteosarcoma Expression Phenotype by MicroRNAs

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## Abstract

***Background:*** Osteosarcomas are the most common primary malignant tumors of bone and show multiple and complex genomic aberrations. miRNAs are non-coding RNAs capable of regulating gene expression at the post transcriptional level, and miRNAs and their target genes may represent novel therapeutic targets or biomarkers for osteosarcoma. In order to investigate the involvement of miRNAs in osteosarcoma development, global microarray analyses of a panel of 19 human osteosarcoma cell lines was performed.

***Principal findings:*** We identified 177 miRNAs that were differentially expressed in osteosarcoma cell lines relative to normal bone. Among these, miR-126/miR-126*, miR-142-3p, miR-150, miR-223, miR-486-5p and members of the miR-1/miR-133a, miR-144/miR-451, miR-195/miR-497 and miR-206/miR-133b clusters were found to be downregulated in osteosarcoma cell lines. All miRNAs in the paralogous clusters miR-17-92, miR-106b-25 and miR-106a-92 were overexpressed. Furthermore, the upregulated miRNAs included miR-9/miR-9*, miR-21*, miR-31/miR-31*, miR-196a/miR-196b, miR-374a and members of the miR-29 and miR-130/301 families. The most interesting inversely correlated miRNA/mRNA pairs in osteosarcoma cell lines included miR-9/TGFBR2 and miR-29/p85α regulatory subunit of PI3K. PTEN mRNA correlated inversely with miR-92a and members of the miR-17 and miR-130/301 families. Expression profiles of selected miRNAs were confirmed in clinical samples. A set of miRNAs, miR-1, miR-18a, miR-18b, miR-19b, miR-31, miR-126, miR-142-3p, miR-133b, miR-144, miR-195, miR-223, miR-451 and miR-497 was identified with an intermediate expression level in osteosarcoma clinical samples compared to osteoblasts and bone, which may reflect the differentiation level of osteosarcoma relative to the undifferentiated osteoblast and fully differentiated normal bone. Significance: This study provides an integrated analysis of miRNA and mRNA in osteosarcoma, and gives new insight into the complex genetic mechanisms of osteosarcoma development and progression.

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## Introduction

MicroRNAs (miRNAs) are small, non-coding RNA molecules that are highly conserved across species and play key roles as regulators of gene expression. miRNAs have been estimated to regulate as much as 60% of the human protein coding genes [1], and modulate the levels of proteins involved in most biological processes, including development, cell proliferation, apoptosis and differentiation.

miRNAs are transcribed as monocistronic or polycistronic stem-loop RNA structures. The polycistronic transcripts contain clusters of several collinear immature miRNAs. After several steps of processing, a miRNA duplex of 20–22 nucleotides is formed from each individual hairpin-forming miRNA transcript. One of the strands of the hairpin loop is thought to be involved in gene regulation, whereas the other, the "passenger strand" or miRNA*, is expected to be less active and more frequently degraded. The active miRNA is preferentially incorporated into the RNA-induced silencing complex (RISC), which recognizes specific mRNA targets through complementary binding, mainly mediated through the "seed" sequence. miRNAs with identical seed sequences may target the same mRNA, and are grouped into miRNA families. On the other hand, the same miRNA may target multiple mRNAs. miRNA targeting causes posttranscriptional gene silencing by inducing mRNA degradation or repressing translation (reviewed in [2]).

Many studies have shown that miRNAs are aberrantly regulated in human cancers, suggesting a role as a novel class of

oncogenes and tumor suppressors. miRNA expression profiles can distinguish tumors from corresponding normal tissues, as well as by their developmental origin and differentiation state [3,4,5].

Osteosarcoma is the most frequent primary malignant bone tumor in children and young adolescents. Survival rates have improved considerable after the introduction of multiagent chemotherapy in the 1980s, with a 5-year survival rate of 60–65% for patients without evidence of metastasis (reviewed in [6]). However, the survival rates have reached a plateau, and further improvements are probably dependent on novel biology-based therapies. At the molecular level, conventional osteosarcomas show complex genomic aberrations and highly variable patterns of gene expression [7]. miRNAs deregulated in human osteosarcoma compared to bone, osteoblasts and mesenchymal stem cells were recently published [8,9,10,11,12]. In addition, a few studies describing expression of selected miRNAs in osteosarcomas [13,14,15] are available, as well as a database of miRNA in sarcoma [16]. Using microarray profiling in an integrative approach, we have analysed genome-wide miRNA and mRNA expression patterns for the EuroBoNeT osteosarcoma cell line panel [17] compared against normal bone. Expression profiles of selected miRNAs were confirmed in clinical samples compared to bone and osteoblasts, providing new insight into the complex genetic mechanisms of osteosarcoma development and progression.

## Materials and Methods

### Ethic Statement

Use of patient samples was according to local medical ethical regulation for each EuroBoNet partner institute. For the Norwegian cohort, the information given to the patients, the written consent used, the collection of samples and the research project were approved by the Regional Ethical Committee for Southern Norway (Project #S-06133).

### Osteosarcoma and Normal Samples

The EuroBoNeT panel of human osteosarcoma cell lines [17] (n = 19) HAL, HOS, 143B, IOR/MOS, IOR/OS9, IOR/OS10, IOR/OS14, IOR/OS15, IOR/OS18, SARG, KPD, MG-63, MHM, MNNG/HOS, OHS, OSA, Saos-2, U-2 OS and ZK-58 were derived from ATCC (www.lgcstandards-atcc.org) or different partner laboratories within EuroBoNeT. The cell lines were grown as previously described [17]. Cell line authentication was performed by STR DNA profiling using Powerplex 16 (Promega, Madison, USA), and the data were validated using the profiles of the EuroBoNeT cell bank [17] and ATCC.

Primary clinical osteosarcoma samples were obtained from the Norwegian Radium Hospital (n = 12), and from a panel collected within EuroBoNeT (n = 71) ([18,19]. Normal long bones were purchased from Capital Biosciences (n = 2) (Maryland, USA) or obtained from amputations of cancer patients at the Norwegian Radium Hospital (n = 4) and University College London (n = 1) where the normal samples were collected distant from the margin of the tumor.

Commercially available primary osteoblast cultures isolated from human calvaria (n = 2) (Sciencell Research Laboratories, California, USA) and from femur and tibia of different donors (n = 3) (Cambrex BioScience, Maryland, USA) were included. The osteoblast cells were maintained in medium provided by the manufacturer, split when reaching 80% confluency, and harvested when enough cells for DNA and RNA isolation were obtained.

An overview of the different cohorts used in the experiments is given in Table S1.

### RNA Isolation

Total RNA, including small RNAs, from osteosarcoma cell lines, clinical samples, bone and osteoblasts was extracted using the Qiagen miRNeasy Mini kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's protocol. Total RNA, not including small RNAs, from osteosarcoma cell lines, clinical samples and bone was extracted using TRIZOL (Invitrogen, California, USA) followed by RNA cleanup using the Qiagen RNeasy Mini kit (Qiagen) with on-column DNase treatment. RNA purity and quantity were measured on a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Delaware, USA), and RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., California, USA).

### miRNA and mRNA Expression Profiling

miRNA expression profiling was performed using the Agilent miRNA Microarray System and the miRNA Complete Labeling and Hyb Kit Version 2.0, and hybridized to Agilent Human miRNA Microarrays (version 2, 799 human miRNAs) following the manufacturer's instructions. miRNA data was imported into GeneSpring GX10 (Agilent), and the intensity values were $\log_2$ transformed and quantile normalized. miRNAs with detectable expression in at least 75% of the bone samples and/or 25% of the osteosarcoma cell lines were retained for further analysis, enabling the identification of miRNAs present in only a subgroup of the cell lines.

cDNA synthesis, cRNA amplification and Illumina Human-6 v2.0 Expression BeadChip hybridization for mRNA expression profiling were performed as previously described [18]. The data was extracted and quality controlled using the Gene Expression module v3.1.7 of Illuminas BeadStudio software (v3.1.0.0). Variance-stabilizing transformation [20] and quantile normalization on the probe level were carried out using the statistical package R [21] and the Bioconductor package lumi [22,23].

MIAME (minimum information about a microarray experiment) compliant data can be downloaded from the GEO repository (www.ncbi.nlm.nih.gov/geo/), accession number GSE28425 for the miRNA and mRNA of the cell lines and bone and GSE30699 for the previously published mRNA data of the clinical samples [19].

### Quantitative Real-time Reverse-transcription PCR

Quantitative real-time reverse-transcription PCR (qRT-PCR) was performed using the ABI PRISM 7500 DNA Sequence Detection System (Life Technologies, California, USA). The TaqMan MicroRNA Reverse Transcription Kit with Megaplex Primer Pool was used to generate cDNA, and TaqMan MicroRNA Assays (Life Technologies) (Table S2) was used to quantitatively detect mature miRNAs. RNU44 and RNU6B were used as endogenous reference genes for normalization. The relative expression levels were determined using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method as described by the manufacturer. For miRNAs with undetectable expression levels in the clinical samples, the Ct-value was set to 40. miRNA qRT-PCR data of clinical samples, bone and osteoblasts were analyzed using Mann-Whitney U test with p-value cut-off of 0.05, and p-values between 0.05 and 0.15 were regarded as showing a trend towards significance.

### Analysis and Integration of miRNA and mRNA Data

Statistical tests were performed to identify miRNAs and mRNAs that were significantly differentially expressed between osteosarcoma cell lines or clinical samples and normal bone; the

miRNA and mRNA genome-wide expression data were imported into GeneSpring GX10 (Agilent) and t-tests were performed applying a Benjamini and Hochberg FDR adjusted p-value cut-off of 0.05.

Predicted mRNA targets containing conserved binding sites for the miRNAs were extracted from the database TargetScan v5.1 (www.targetscan.org) using GeneSpring GX10. Targets for the miRNA* were predicted using TargetScan custom v5.1. Pearson's correlation ($r$) was calculated for each miRNA and its predicted mRNA targets across all the osteosarcoma cell line samples. miRNA- mRNA pairs with $r < -0.5$ were selected for further analysis. Selected predicted targets were imported into MetaCore™ (GeneGo, Michigan, USA) in order to identify pathways, biological functions and molecular interactions of the candidate target genes. The top significant enriched pathways and networks are presented, given a p-value significance level with a false discovery rate (FDR) multiple testing correction based on the Simes modification of the Bonferroni procedure [24].

## Results

### Global miRNA and mRNA Expression Patterns

After processing of the miRNA expression profiles of 19 EuroBoNeT human osteosarcoma cell lines and four bone samples, 340 miRNAs were identified as expressed in at least 25% of the cell lines and/or 75% of the normal bone samples. mRNA profiles were also obtained, and distinct miRNA or mRNA patterns could be identified that distinguished certain subclasses of the osteosarcoma cell lines, clearly distinct from the pattern of normal bone. The main class determined by miRNA pattern was also identified by mRNA pattern, indicated in black in Figure 1 whereas the two other subclasses were intermixed. U2OS separates for the main subclusters, but the difference in branch length is marginal.

### Deregulated miRNAs in Osteosarcoma Cell Lines

A statistical analysis identified 177 miRNAs, including 38 miRNA*s, that were significantly differently expressed between osteosarcoma cell lines and normal bone. All but 10 of the miRNAs had a more than two-fold change of intensity, 103 were upregulated and 74 were downregulated in the osteosarcoma cell lines compared to the mean expression level of the bones (Table S3). The top 10 up- and downregulated miRNAs are listed in bold in Table 1.

Of these 177 identified miRNAs, 90 belonged to evolutionarily conserved miRNA families as defined by Friedman et al. [1], indicating functional conservation and important roles. Further, among these, 43 miRNAs (50%) belonged to common conserved families or were transcribed together from polycistronic miRNA clusters. Deregulated miRNA clusters have been shown to be significantly overrepresented compared to single miRNAs in most investigated diseases [25]. The 43 miRNAs are included in Table 1 and annotated with miRNA family or cluster relation. These groups of miRNAs showed comparable up- or downregulation, and a concerted action could ensure a synergistic, redundant and more flexible means of regulation and have a higher potential to influence complex cell signalling networks.

The highly downregulated miRNAs presented in Table 1 were miR-126/miR-126*, miR-142-3p, miR-150, miR-223, miR-363, miR-486-5p and members of the miR-1/miR-133a, miR-206/miR-133b, miR-451/miR-144 and miR-497/miR-195 clusters. The miR-17-92 and the paralogous miR-106b-25 and miR-106a-92 clusters encode 12 mature miRNAs of which all were significantly overexpressed in the osteosarcoma cell lines. Furthermore, the overexpressed miRNAs included miR-7, miR-9/miR-9*, miR-21*, miR-31/miR-31*, miR-181, miR-196a/miR-196b, miR-503 and members of the miR-29 and miR-130/301 families (Table 1).

A global expression profile describing deregulated miRNAs in human osteosarcoma clinical samples compared to osteoblasts was recently published [8]. Among the list of 38 deregulated miRNAs identified by Maire et al., 16 miRNAs were also found to be deregulated in our cell lines and all but three miRNAs were found to be oppositely regulated in these two studies. Focusing on the conserved miRNAs presented in Table 1, we found that of the 14 miRNAs downregulated in our study relative to normal bone, six were published as upregulated in osteosarcoma relative to osteoblasts, namely the miRNAs miR-126, miR-142-3p, miR-195, miR-223, miR-451 and miR-497, while miR-31/miR-31* was upregulated compared to bone and downregulated compared to osteoblasts. To verify these findings, microarray expression data for osteoblasts were included for these miRNAs, and all, except for miR-223, showed the same expression pattern in our cell lines compared to osteoblasts as in the published clinical data (Figure 2).

### Validation of miRNA Expression in Clinical Samples

In order to verify the above findings in clinical samples, a smaller cohort of 12 clinical samples, six normal bone and five osteoblasts was collected. A subset of 15 miRNAs from Table 1 was selected for confirmation by qRT-PCR (Figure 3). Interestingly, all but two miRNAs showed an intermediate expression level in osteosarcoma clinical samples compared to the mean values in osteoblasts and bone, confirming the trend that was already observed in the cell lines. These 13 miRNAs include all the above seven miRNAs (omitting miR-31*) previously described in osteoblasts [8] as well as miR-1, miR-18a, miR-18b, miR-19b, miR-133b and miR-144.

Of the 15 miRNAs, all but miR-19b showed significant changes in gene expression level for clinical samples compared to either bone and/or osteoblasts. The expression pattern in cell lines compared to bone was confirmed in the clinical samples for 13 of 15 miRNAs. The level of change was significant for nine of these miRNAs; miR-1, miR-9, miR-18a, miR-18b, miR-126, miR-133b, miR-144, miR-195 and miR-223. miR-451 and miR-497 showed a trend towards being significantly decreased, miR-31 showed a heterogenous expression pattern, and miR-19b, miR-29b and miR-142-3p were expressed at comparable level in clinical samples and bone. These last miRNAs, miR-451, miR-31, miR-142-3p and miR-29b, as well as miR-1, miR-9, miR-126, miR-144, miR-195, miR-223 and miR-497, were all significantly different in clinical samples compared to osteoblasts. miR-133b showed a trend towards being significantly decreased, being undetected in two clinical samples and all osteoblasts. miR-144 was undetected in all osteoblasts, and miR-1 and miR-451 was undetected in two and three of the osteoblast samples, respectively.

### Expression of miR-17-92, miR-106b-25 and miR-106a-92 Clusters and Correlation with mRNA Host Genes

The miRNAs of the miR-17-92, miR-106b-25 and miR-106a-92 clusters had similar expression patterns in the osteosarcoma cell lines, although the miRNAs from the miR-106b-25 cluster showed slightly lower correlation with the other miRNA clusters (Figure 4). miR-18a of the miR-17-92 cluster and miR-18b of the miR-106a-92 cluster showed almost identical expression patterns in clinical samples, while some divergence was observed for miR-19b being encoded by both clusters (Figure 3). The more highly expressed miRNA*s in osteosarcoma cell lines

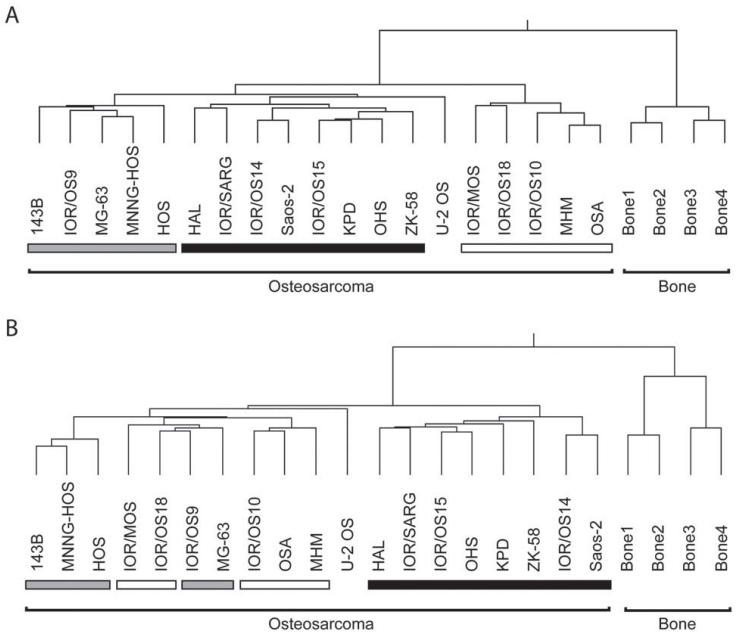


**Figure 1. Unsupervised hierarchical clustering of 19 osteosarcoma (OS) cell lines and four normal bone samples. A**. 361 miRNAs expressed in at least 75% of the bone samples and/or 25% of the osteosarcoma cell lines. **B**: Expression of the 48,701 mRNA probes. Distance metric: Pearson's correlation absolute average. Bars under cell lines indicate samples with similar expression profile in miRNA (A) and mRNA (B) data.
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showed high correlation with the miRNA of the complementary strand, as exemplified by miRNA/miRNA*s of the miR-106b-25 and miR-17-92 clusters (miRNA* in Figure 4B).

The miR-17-92 and miR-106b-25 clusters are encoded within introns of the mRNAs *c13orf25* and *MCM7*, respectively, and showed high positive correlation ($r = 0.6–0.9$) with the expression level of the host genes (Figure 4). *MCM7* and miR-106b-25, located at 7q22.1, were amplified in many of the cell lines (58%), while *c13orf25* and the miRNA cluster miR-17-92, located at 13q31.2, were amplified in only a minority of the cell lines and hemizygously deleted in 32% [26]. Despite the different patterns of amplification and deletions of these paralogous miRNA clusters, the expression levels of the miRNAs were strikingly similar. Thus, the copy number changes in the regions harboring these miRNAs seem to be overridden by other regulatory mechanisms.

The transcription factors E2F1, E2F2, E2F3 and MYC activate the members of the miR17-92 clusters [27,28,29], and MYC and E2F1 have also been shown to regulate the miR-106b-25 cluster [30]. *MYC*, *E2F1* and *E2F3* were amplified in a high number of the cell lines (74%, 68%, 47% and 42%, respectively) [26], and was also shown to be amplified in a set of 29 clinical samples (52%, 7%, 34% and 24%, respectively) [31]. However, no correlations ($r<0.5$) were observed between the expression levels of *E2F1*, *E2F2*, *E2F3* and *MYC* and the mature miRNAs of the three paralogous miRNA clusters miR-106b-25, miR-17-92 and miR-106a-92, neither at the RNA or protein level (not shown).

## mRNA Expression in Osteosarcoma Cell Lines and Clinical Samples

Statistical analysis identified 8,982 mRNA probes that were significantly differently expressed between the osteosarcoma cell lines and normal bone. About 50% of these genes (4,256 mRNAs) were confirmed to be changed also in clinical samples, being significantly differently expressed between 71 osteosarcoma clinical samples and normal bone. A hypergeometric test showed that the large overlap between the two datasets was highly significant with a p-value $<1.0E–1000$.

Analyses of functional annotation were performed on these gene sets using GeneGo, and the top 10 enriched processes in each of the sample categories were listed. Despite the observation that expression of numerous genes was unique to clinical or cell line samples, enrichment for similar processes like calcium signaling, apoptosis and cell differentiation was observed irrespective of sample origin (Table S4).

## Identification of Candidate miRNA Targets

An integrated analysis was performed on the miRNA and mRNA data to identify candidate miRNA targets. Several levels of filtering were applied to the lists of significantly differently expressed miRNAs and mRNAs. Firstly, predicted mRNA targets for the 90 conserved miRNAs were identified, as well as targets for the 29 miRNA*s encoded in the stem-loop sequence of miRNAs belonging to phylogenetically conserved families. These mRNA targets were filtered to only contain significantly differently expressed mRNA genes common to cell lines and clinical samples, thus avoiding *in vitro* artifacts. Secondly, the Pearson's correlation

**Table 1.** Down- and upregulated miRNAs in osteosarcoma cell lines versus bone.

| miRNA | miR family | miR cluster | Fold change | Presence CL/Bone |
|---|---|---|---|---|
| **Downregulated** | | | | |
| **miR-451** | | miR-451/miR-144 | −28000 | 1/4 |
| **miR-1** | miR-1/206 | miR-1/miR-133a | −550 | 4/All |
| **miR-144/miR-144*** | | miR-451/miR-144 | −280/−87 | 0/3 |
| **miR-223** | | | −240 | 0/All |
| **miR-142-3p** | | | −190 | 7/4 |
| **miR-133b** | miR-133 | miR-206/miR-133b | −86 | 4/All |
| **miR-150** | | | −84 | 0/4 |
| **miR-206** | miR-1/206 | miR-206/miR-133b | −81 | 0/All |
| **miR-126/126*** | | | −65/−30 | 17/4 (1/4) |
| **miR-486-5p** | | | −42 | 5/4 |
| miR-195 | miR-15/16/195/424/497 | miR-195/miR-497 | −24 | All |
| miR-497 | miR-15/16/195/424/497 | miR-195/miR-497 | −22 | 12/4 |
| miR-363 | miR-25/32/92/92ab/363/367 | | −14 | 6/All |
| miR-133a | miR-133 | miR-1/miR-133a | −9.2 | 3/4 |
| **Upregulated** | | | | |
| **miR-18a** | miR-18 | miR-17-92 | 95 | All/2 |
| **miR-9/miR-9*** | | | 65/42 | 18/0 |
| **miR-301a** | miR-130/301 | | 47 | All/2 |
| **miR-18b** | miR-18 | miR-106a-92 | 47 | All/1 |
| **miR-31/miR-31*** | | | 35 | 14/1 (14/0) |
| **miR-503** | | | 27 | 18/0 |
| **miR-301b** | miR-130/301 | miR-301b/130b | 23 | All/0 |
| **miR-21*** | | | 22 | 18/1 |
| **miR-7** | | | 21 | 18/1 |
| **miR-137** | | | 19 | 10/0 |
| miR-96 | | miR-96/miR-182 | 18 | All/2 |
| miR-130b | miR-130/301 | miR-301b/130b | 14 | All/1 |
| miR-19a | miR-19 | miR-17-92a | 13 | All |
| miR-196a | miR-196 | | 10 | All/2 |
| miR-542-3p | | miR-542/miR-450a | 8.3 | All/2 |
| miR-29b | miR-29 | miR-29b-2/miR-29c | 7.1 | All |
| miR-106a | miR-17-5p/20/93/106/519 | miR-106a-92 | 7.1 | All/2 |
| miR-32 | miR-25/32/92/92ab/363/367 | | 6.5 | All/1 |
| miR-421 | | miR-421/374b | 6.1 | All/1 |
| miR-17 | miR-17-5p/20/93/106/519 | miR-17-92a | 5.8 | All |
| miR-99b | | miR-99b/let7/miR-125a | 5.7 | All |
| miR-20a | miR-17-5p/20/93/106/519 | miR-17-92a | 5.5 | All |
| miR-125a-5p | | miR-99b/let7/miR-125a | 5.3 | All |
| miR-93 | miR-17-5p/20/93/106/519 | miR-106b-25 | 4.9 | All |
| miR-19b | miR-19 | miR-17-92/miR-106a-92 | 4.8 | All |
| miR-106b | miR-17-5p/20/93/106/519 | miR-106b-25 | 4.5 | All |
| miR-182 | | miR-96/miR-182 | 4.5 | 12/0 |
| miR-450a | | miR-542/miR-450a | 4.4 | All/1 |
| miR-20b | miR-17-5p/20/93/106/519 | miR-106a-92 | 4.3 | All |
| miR-196b | miR-196 | | 4.3 | All |
| miR-374a | miR-374 | | 4.0 | All |
| let-7e | | miR-99b/let7/miR-125a | 3.8 | All |
| miR-181b | miR-181 | | 3.6 | All |

**Table 1.** Cont.

| miRNA | miR family | miR cluster | Fold change | Presence CL/Bone |
|---|---|---|---|---|
| miR-374b | miR-374 | miR-421/374b | 3.3 | All |
| miR-103 | miR-103/107 | | 2.9 | All |
| miR-181d | miR-181 | | 2.7 | All/3 |
| miR-29a | miR-29 | miR-29a/miR-29b-1 | 2.5 | All |
| miR-25 | miR-25/32/92/92ab/363/367 | miR-106b-25 | 2.4 | All |
| miR-92a | miR-25/32/92/92ab/363/367 | miR-17-92/miR-106a-92 | 2.3 | All |
| miR-107 | miR-103/107 | | 2.1 | All |

The table lists 43 conserved miRNAs that are transcribed together from polycistronic miRNA clusters and/or belong to the same conserved families. In addition the top10 miRNAs are included (bold), of which some belong to the common families or clusters. miRNAs with common features are annotated with miRNA family and/or cluster members. For miRNAs detected as present in only a minority of the samples, the fold change (mean of cell lines vs bone) could be overestimated. Cell lines (CL), n = 19; bone, n = 4.

doi:10.1371/journal.pone.0048086.t001

for each of the miRNAs and its predicted targets across all the cell line samples was calculated. Pairs of miRNA or miRNA* and their predicted mRNA targets that did not show significant negative correlation ($r > -0.5$) were removed from the data set, resulting in a set of 199 pairs represented by 67 miRNA/miRNA* and 116 target mRNAs (Table S5). Analysis of functional annotation of these 116 mRNAs was performed, and the genes were found to be enriched in processes involved in apoptosis, calcium signaling and regulation of cell cycle, as well as pathways involving cytoskeleton remodeling, HIF regulation, PI3K pathway and development through IGF-1 and EGFR signaling (Table S4). Of the 15 miRNAs confirmed to be significantly differently expressed in clinical samples compared to either bone or osteoblasts, 8 miRNAs showed inverse correlation with at least one target mRNA, and these 30 mRNAs are highlighted in bold in Table S5.

The 116 mRNA targets were ranked based on the level of conservation of each of the miRNA target sites. A probability of preferentially conserved targeting ($P_{CT}$) value has been calculated for all highly conserved miRNA families, where the $P_{CT}$ value reflects the probability that a site is conserved due to selective maintenance of miRNA targeting rather than by chance or any other reason not pertinent to miRNA targeting [1]. miRNA/mRNA pairs with $P_{CT} < 0.4$ were then removed from the dataset, but the miRNA*s were contained as no information regarding $P_{CT}$ exists for these miRNAs, reducing the number of targets to 72 mRNAs. Twenty-six of the mRNAs showed an inverse correlation with at least two miRNAs each, mainly from the same miRNA family. These 80 pairs of 38 miRNA/miRNA*s and 26 mRNAs may identify the most interesting mRNA targets (Table 2).

Among these targets is transforming growth factor beta receptor II (*TGFBR2*), highly and significantly downregulated in both cell lines and clinical samples. The correlation plot of *TGFBR2* and miR-9 is shown in Figure S1. In the cell lines, miR-29a and miR-29b were inversely correlated with numerous interesting predicted targets like phosphoinositide-3-kinase regulatory subunit 1 (*PIK3R1/p85α*) and phosphoinositide-3-kinase regulatory subunit 3 (*PIK3R3/p55γ*), both central members of the PI3K/PTEN/Akt pathway. Insulin receptor substrate-1 (*IRS-1*), a downstream target of Akt, inversely correlated with miR-7 and miR-181b. In addition, the expression of the tumor suppressor gene phosphatase and tensin homolog (*PTEN*) inversely correlated with miR-17, miR-20b, miR-9* and miR-92a (Table 2), but also showed a

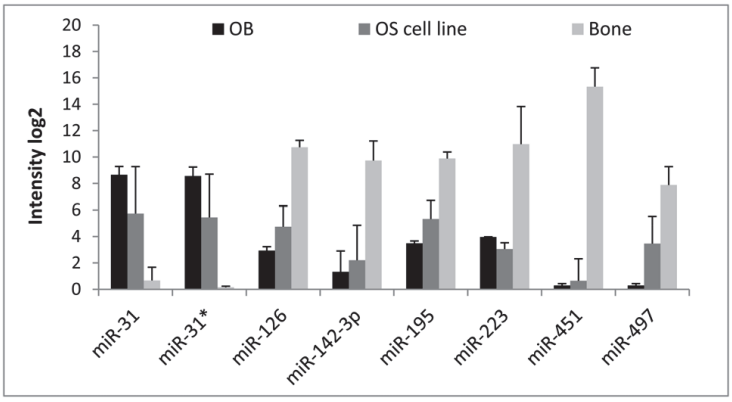
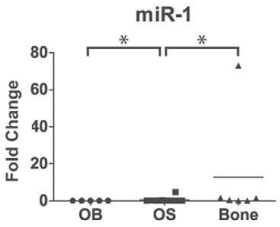
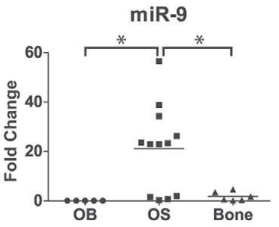
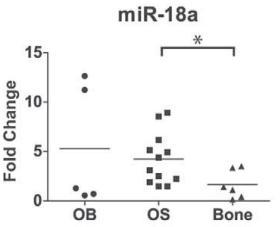


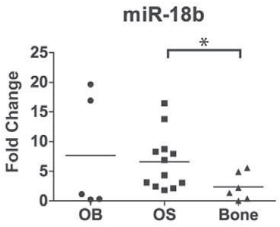

**Figure 2. miRNA microarray expression levels in osteosarcoma cell lines, normal bone and osteoblasts.** The expression level is shown as a mean for each group of samples; two osteoblast primary cultures, 19 osteosarcoma cell lines and four bones. OS: osteosarcoma; STDEV: standard deviation.

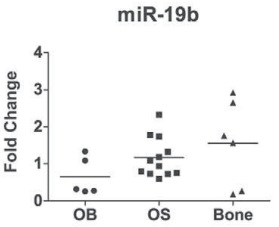
doi:10.1371/journal.pone.0048086.g002

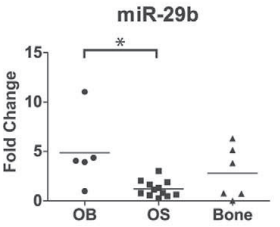

miR-1
Fold Change
80
60
40
20
0
OB
OS
Bone
*
*

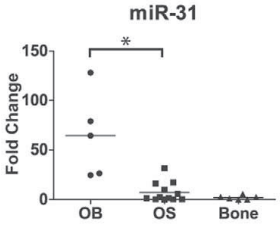

miR-9
Fold Change
60
40
20
0
OB
OS
Bone
*
*

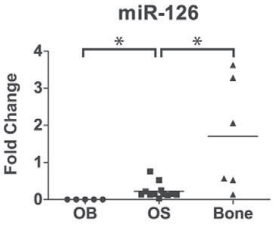

miR-18a
Fold Change
15
10
5
0
OB
OS
Bone
*

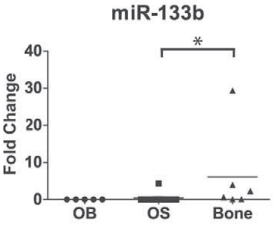

miR-18b
Fold Change
25
20
15
10
5
0
OB
OS
Bone
*

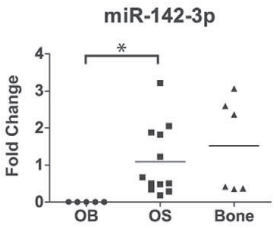

miR-19b
Fold Change
4
3
2
1
0
OB
OS
Bone

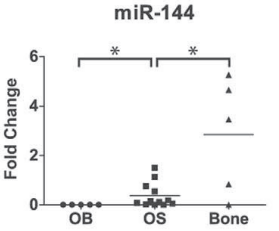

miR-29b
Fold Change
15
10
5
0
OB
OS
Bone
*

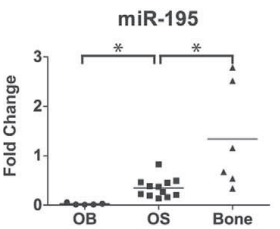

miR-31
Fold Change
150
100
50
0
OB
OS
Bone
*

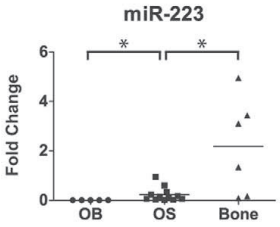

miR-126
Fold Change
4
3
2
1
0
OB
OS
Bone
*
*

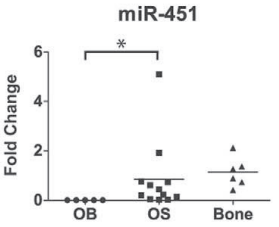

miR-133b
Fold Change
40
30
20
10
0
OB
OS
Bone
*


miR-142-3p
Fold Change
4
3
2
1
0
OB
OS
Bone
*


miR-144
Fold Change
6
4
2
0
OB
OS
Bone
*
*


miR-195
Fold Change
3
2
1
0
OB
OS
Bone
*
*


miR-223
Fold Change
6
4
2
0
OB
OS
Bone
*
*


miR-451
Fold Change
6
4
2
0
OB
OS
Bone
*

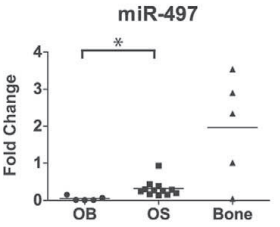

miR-497
Fold Change
4
3
2
1
0
OB
OS
Bone
*

**Figure 3. Verification of expression levels of miRNAs in osteosarcoma clinical samples, normal bone and osteoblasts.** Scatter plot showing the expression level of 15 miRNAs, generated from qRT-PCR data. The expression values are relative to the mean expression level of bone samples. *: p-value <0.05; vertical line: mean value.
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modest inverse correlation ($r = -0.4$ to $-0.5$) with other miRNAs of the miR-17, miR-19, miR-130/301 and miR-26 families (Table S6).

## Discussion

Osteosarcomas show complex genomic changes with few recurrent chromosomal aberrations, which make it difficult to identify the molecular features that underlie the development of this type of cancer. As an increasing amount of high-throughput data from different genomic levels is being generated, the possibility of integrating these data gives us the unique opportunity to identify important genetic and epigenetic alterations in osteosarcoma. Among these, miRNAs and their target genes may represent potential novel therapeutic targets or biomarkers for osteosarcoma. In this study, we have identified deregulated miRNAs in osteosarcoma, and used the theoretical knowledge of predicted miRNA and mRNA interactions combined with experimental data to identify the most likely candidate mRNA targets and pathways.

Both miRNA and mRNA expression patterns clearly distinguished osteosarcoma cell lines from normal bone, as well as identified distinct subsets of samples. Since miRNA expression pattern might represent a certain cellular state and would impact on the mRNA transcriptome, one might expect that mRNA profiles are modulated by the miRNA patterns, so that subgroups defined by either would be overlapping. As shown, this was indeed the case, as the main osteosarcoma subcluster was identical whether based on miRNA or mRNA expression. Even though the other subclusters were less well maintained, as their branches were intermingled, this confirms the connection between miRNA patterns and the expressed transcriptome. No distinct phenotypic grouping according to previously identified properties of the cell lines was observed [17,32]; rather these subsets reflect some other characteristic which is visible at both the miRNA and mRNA level.

A global expression profile describing the deregulation of 38 miRNAs in human osteosarcoma clinical samples compared to osteoblasts was recently published [8]. Of these, 16 miRNAs were also found to be deregulated in our cell panel. Interestingly, all but three miRNAs showed opposite regulation between these two studies, being upregulated in osteosarcoma clinical samples relative to osteoblasts and downregulated in our cell lines relative to normal bone and vice versa. To resolve these observations,

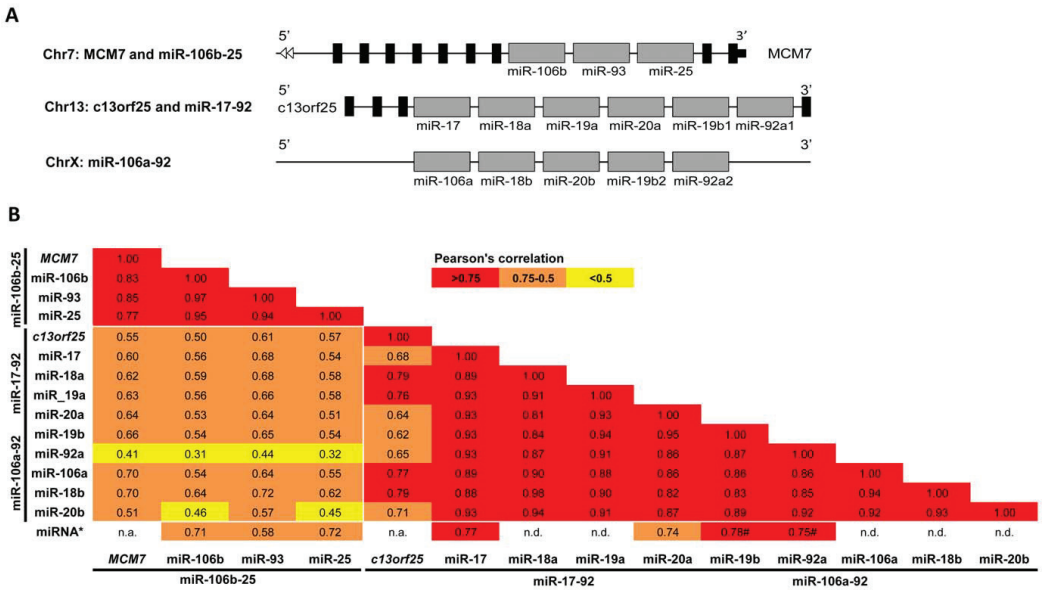


**Figure 4. Genomic organization and Pearson's correlation of miR-106b-25, miR-17-92, miR-106a-92 and mRNA host genes MCM7 and c13orf25.** A. Schematic illustration of the genomic organization of miRNA clusters and host genes. *MCM7* and miR-106b-25 are located on 7q22.1, *c13orf25* and miR-17-92 are located on 13q31.2 and the miR-106a-92 cluster on Xq26.2. Black and grey boxes indicate exons and miRNAs, respectively, not in scale. B. Pearson's correlation between the miRNAs of the miR-106b-25, miR-17-92 and miR-106a-92 clusters and the host genes *MCM7* and *c13orf25*. miR-17-92 and miR-106a-92 contains common mature miRNAs, hence the two clusters are not separated. miRNA* represents the correlation between the miRNA of the first strand and the second strand of the hairpin, ex between miR-17 and miR-17*. The values are colour-coded according to Pearson's correlation in cell lines. #: Pearson's correlation only calculated for miR-19b-1* and miR-92a-1* since miR-19b-2* and miR-92a-2* were not detected. n.d.: miRNA not detected in most of the samples due to low expression levels.
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**Table 2.** Potential target mRNAs of identified miRNAs.

| Symbol | RefSeq ID | Name | miRNA |
|---|---|---|---|
| AKAP13 | NM_006738.4 | A kinase (PRKA) anchor protein 13 | miR-17, miR-20a, miR-93, miR-106a, miR-106b |
| ARHGAP24 | NM_001025616.1 | Rho GTPase activating protein 24 | miR-133a, miR-133b, miR-590-5p |
| CPEB3 | NM_014912.3 | cytoplasmic polyadenylation element binding protein 3 | miR-29a, miR-29b |
| IRS2 | NM_003749.2 | insulin receptor substrate 2 | miR-7, miR-181b |
| KCNJ10 | NM_002241.2 | potassium inwardly-rectifying channel, subfamily J, member 10 | miR-93, miR-106b |
| KIAA1632 | NM_020964.1 | KIAA1632 | miR-25, miR-32 |
| LIMK1 | NM_016735.1 | LIM domain kinase 1 | miR-20a, miR-20b, miR-106a |
| MEX3B | NM_032246.3 | mex-3 homolog B (C. elegans) | miR-29a, miR-29b |
| MPP1 | NM_002436.2 | membrane protein, palmitoylated 1, 55kDa | miR-32, miR-25 |
| MTF1 | NM_005955.1 | metal-regulatory transcription factor 1 | miR-25, miR-130b |
| PIK3R1 | NM_181504.2 | phosphoinositide-3-kinase, regulatory subunit 1 (alpha) | miR-29a, miR-29b, miR-96 |
| PIK3R3 | NM_003629.2 | phosphoinositide-3-kinase, regulatory subunit 3 (p55, gamma) | miR-21*, miR-29a, miR-29b |
| PTEN | NM_000314.3 | phosphatase and tensin homolog (mutated in multiple advanced cancers 1) | miR-17, miR-20b, miR-9*, miR-92a |
| RHOC | NM_175744.3 | ras homolog gene family, member C | miR-17, miR-20a, miR-20b, miR-106a |
| SAPS2 | XM_942540.1 | PREDICTED: SAPS domain family, member 2 | miR-20b, miR-106a |
| SLC25A37 | NM_016612.1 | solute carrier family 25, member 37 (SLC25A37) | miR-181b, miR-181d |
| SLC2A3 | NM_006931.1 | solute carrier family 2 (facilitated glucose transporter), member 3 | miR-195, miR-497 |
| SNX4 | NM_003794.2 | sorting nexin 4 | miR-29a, miR-29b |
| SOX5 | NM_006940.4 | SRY (sex determining region Y)-box 5 | miR-195, miR-497, miR-503 |
| SYT7 | NM_004200.2 | synaptotagmin VII | miR-17, miR-20a, miR-20b, miR-93, miR-106b |
| TGFBR2 | NM_001024847.1 | transforming growth factor, beta receptor II (70/80kDa) | miR-9, miR-590-5p |
| TNFRSF1B | NM_001066.2 | tumor necrosis factor receptor superfamily, member 1B | miR-19a, miR-19b, miR-148b, miR-301a, miR-301b |
| WDR40A | NM_015397.1 | WD repeat domain 40A | miR-29a, miR-29b |
| ZBTB47 | NM_145166.2 | zinc finger and BTB domain containing 47 | miR-7-1*, miR-17, miR-18a, miR-18b, miR-19a, miR-19b, miR-20a, miR-20b, miR-106a |
| ZFP91 | NM_170768.1 | zinc finger protein 91 homolog (mouse) | miR-17, miR-19a, miR-19b, miR-20a, miR-20b |
| ZNF385D | NM_024697.1 | zinc finger protein 385D | miR-32, miR-92a |

miRNA with more than one binding site for the mRNA.

The mRNAs are differently expressed in osteosarcoma cell lines and clinical samples and predicted to be targeted by several of the differently expressed miRNAs. The pairs of miRNAs and mRNA show a Pearson's correlation ($r < -0.5$) in cell lines.

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seven of these miRNAs as well as eight additional deregulated and phylogenetic conserved miRNAs from the osteosarcoma cell line analysis were selected for validation in clinical samples. As predicted, the 13 miRNAs miR-1, miR-18a, miR-18b, miR-19b, miR-31, miR-126, miR-133b, miR-142-3p, miR-144, miR-195, miR-223, miR-451 and miR-497 showed opposite regulation when the osteosarcoma clinical samples were compared against bone or osteoblasts. The inverse deregulation of miRNAs compared to bone or osteoblasts is consistent with previous publications, Jones et al. [10] identified miR-126, miR-142-5p, miR-195, miR-223 and miR-451 to be downregulated in osteosarcoma versus bone while Lulla et al. [11] reported a subset of these, miR-126, miR-142-3p, miR-223 and miR-451, to be upregulated when compared to osteoblasts. Osteosarcoma has been regarded to develop as a result of genetic changes occurring during the determination or maturation of mesenchymal stem cells or committed progenitor cells (reviewed in [33,34]). The miRNAs may play a role in regulating osteogenesis by the controlled temporal expression of different miRNA as differentiation proceeds, and thus the miRNA expression pattern observed in osteosarcoma may reflect a specific stage of differentiation. Similar modes of regulation have been described for miR-126/miR-126*, miR-223 and miR-451 in erythroid differentiation (reviewed in [34,35]. Osteosarcomas express these miRNAs at an intermediate level compared to osteoblasts and bone, which may be consistent with the differentiation status of osteosarcoma relative to the undifferentiated osteoblasts and fully differentiated bones.

The deregulation of a number of miRNAs in human osteosarcoma compared to bone was recently reported. Thayanithy et al. [12]. and Jones et al. [10] reported 36 miRNAs and 34 miRNAs, respectively, to be deregulated in osteosarcoma clinical samples when compared to bone. The overlap between these two studies was marginal as only the downregulation of miR-150 was observed in both datasets. miR-150 acts as a tumor suppressor in malignant lymphoma through activation of the PI3K-Akt pathway [36]. Using a less stringent cut-off, we identified 177 deregulated

miRNAs in osteosarcoma cell lines, of which 17/36 miRNAs and 16/34 miRNAs from the above studies were confirmed to be significantly changed, including the common miR-150. All but three of these miRNAs showed similar up/down-regulation as previously reported, further supporting our findings.

Previous studies have reported deregulation of specific miRNAs in osteosarcomas (reviewed in [37]). miR-143, down-regulated in osteosarcoma cell lines and primary tumor samples, promotes apoptosis, suppresses tumorigenicity [14] and regulates metastasis [13]. However, the downregulation of miR-143 in our osteosarcoma cell lines was not significant. miR-21 has been reported as overexpressed in a range of different tumors, including osteosarcoma tissue where it is involved in cell invasion and migration [15]. miR-21 was highly expressed in both our tumor and normal samples, whereas miR-21* was among the top 10 overexpressed miRNAs in our tumors.

Expression profiles of selected miRNAs were validated in clinical samples, both to avoid possible artefacts that may have arisen during *in vitro* culturing, or due to regulation reflecting the *in vitro* growth conditions. Confirming our *in vitro* findings, 13/15 miRNAs showed the same expression pattern in clinical samples compared to bone. The only exceptions were miR-19b and miR-29b, upregulated in cell lines while a not significant downregulation was observed in clinical samples. Downregulation of miR-29b in osteosarcoma compared to bone is consistent with a recent report [10]. The level of change was significant for nine of these 13 miRNAs. All of the miRNAs that were confirmed downregulated in clinical samples compared to bone are known to act as tumor suppressors in other types of cancers, that is miR-1, miR-126/miR-126*, miR-133b, miR-144, miR-195, miR-223 and miR-497 [38,39,40,41,42,43].

miR-133b was expressed at low or undetectable level in most of the clinical samples, and was the strongest downregulated miRNA compared to bone. miR-133b was not detected in the osteoblasts. miR-133a/miR-133b have a dual role being essential for myogenesis and suppressing osteogenesis through targeting of runt-related transcription factor 2 (*RUNX2)*, and are downregulated in BMP2-induced osteogenesis of premyoblast mesenchymal cells [44]. RUNX2 is a master regulator of osteogenic differentiation, and the expression level increases gradually during osteogenesis with highest levels observed in early osteoblasts, but then decreases to very low levels in mature osteocytes [45]. Accordingly, *RUNX2* was significantly higher expressed in our osteosarcoma clinical samples, relative to normal bone (results not shown) as has been shown for the comparison of osteosarcoma tumors with osteoblasts [46]. RUNX2 expression has also been detected in most osteosarcoma primary tumors [47]. *RUNX2* does not seem to be reduced at the mRNA level by miR-133 in our study, which may be explained by the high amplification frequency (68%) of *RUNX2* observed in our cell lines, as previously reported [46].

All the miRNAs of the miR-17-92 cluster (13q31.2) and the paralogous miR-106b-25 (7q22.1) and miR-106a-92 clusters (Xq26.2) were highly expressed in osteosarcoma cell lines compared to normal bone, and all showed a similar expression pattern. The upregulation of miR-18a and miR-18b were verified in clinical samples. High levels and amplification of the miR-17-92 and miR-106b-25 clusters have been reported for a multitude of different cancers [5,30,48]. In two recent publications, miR-17-92 [9,12] and miR-106a and miR-106b from the miR-106a-92 and miR-106b-25 clusters [9] were reported upregulated in osteosarcomas. In addition, haploinsuffiency of the miR-17-92 cluster is responsible for developmental defects in individuals with skeletal abnormalities, being the first example where a germline mutation of a miRNA cause hereditary disease [49].

Despite previous reports of amplification of the chromosomal regions containing the miR-106b-25 and miR-17-92, copy number changes were infrequent for these regions in osteosarcoma, strongly indicating that the expression levels are not driven by amplification. As one might expect, there was clear covariation of the host genes *MCM7* and *c13orf25* and their respective intronic miRNA clusters miR-106b-25 and miR-17-92, thus a common regulatory mechanism for both the miRNAs and their host genes seems likely, plausibly due to co-transcription from the mRNA promoter. It was recently shown that the majority of co-expressed miRNAs were under coordinated control by common transcription factors, and were more likely to be functionally related [50]. Previous reports have shown that the transcription factors MYC, E2F1, E2F2 and E2F3 activate the miR-17-92 cluster, and E2F1 has also been shown to activate the miR-106b-25 cluster (reviewed in [51]). E2F1-3 and MYC were frequently amplified in osteosarcoma cell lines and clinical samples [26,31], but no correlation could be observed between the paralogous miRNAs and RNA or protein levels of E2F1-3 and MYC. However, a regulatory network was recently described in osteosarcoma, demonstrating that miRNAs of the 14q32 locus act cooperatively to destabilize MYC and thus control the expression of the miR-17-92 cluster [12].

A number of target genes regulated by the miR-106b-25, miR-17-92 and miR-106a-92 clusters have been reported, such as E2F1-3, cyclin-dependent kinase inhibitor (CDKN1A/p21), BCL2-like 11 (BCL2L11/Bim), RB1 and components of the TGF-β pathway [5,30,52]. miR-9 was highly expressed in osteosarcoma clinical samples and cell lines relative to normal tissue as has been observed for breast cancer [53]. In osteosarcoma cell lines, miR-9 inversely correlated with the predicted target gene transforming growth factor, beta receptor II (*TGFBR2*). An increase of miR-9 and reduced levels of *TGFBR2* was confirmed in osteosarcoma clinical samples. In line with this, overexpression of miR-106b or miR-93, as observed for osteosarcoma, renders gastric cancer cells insensitive to TGFB-mediated cell cycle arrest [30], consistent with an oncogenic role of the miR-106b-25 cluster.

Strong inverse correlation was observed between the tumor suppressor *PTEN* and several members of the miR-17, miR-19, miR-130/301 and miR-26 families, which were upregulated in the osteosarcoma cell lines. *PTEN* was downregulated in both osteosarcoma cell lines and clinical samples, and is a verified target of miR-26a and members of the miR-106b-25 and miR-17-92 clusters [54,55,56,57]. PTEN antagonizes signaling through the PI3K/PTEN/Akt pathway, which plays a crucial role in tumorigenesis by promoting cell proliferation and inhibiting apoptosis. This pathway may also be affected by other osteosarcoma miRNAs, as the p85α regulatory subunit of PI3K showed strong inverse correlation with miR-29a and miR-29b in the cell lines. p85α is a verified target of the miR-29 family [58]. miR-29b was downregulated, although not at a significant level, in osteosarcoma clinical samples compared to bone, consistent with previous reports [10]. A positive correlation has been observed between expression of PTEN and the degree of differentiation in osteosarcoma specimens, and higher expression of PTEN was observed in benign lesions of bone than in osteosarcoma [59]. miRNAs are regarded as fine-tuners of gene expression, and slight variations in the amount of PTEN may contribute to the development of cancers (reviewed in [60]).

## Conclusions

A high number of deregulated miRNAs were identified in osteosarcoma, many of them known to have a role as tumor suppressors or oncogenes in other cancers. A number of identified miRNAs showed opposite regulation when compared to bone or osteoblasts. This observation suggests that osteosarcomas may represent a partially differentiated cell between the undifferentiated osteoblasts and a fully differentiated bone. Deregulated mRNA targets and pathways, like members of the PI3K/PTEN/Akt and TGFB pathway, are involved in important functions related to tumorigenicity, and play an important role in the development of osteosarcoma.

## Supporting Information

**Figure S1 Inverse correlation between *TGFBR2* and miR-9 in cell lines and normal bones.**
(PDF)

**Table S1 Overview of cell lines, clinical samples and normal bone used in the study.**
(XLS)

**Table S2 Overview of TaqMan assays for quantitative real-time PCR experiments.**
(XLS)

**Table S3 Overview of 177 miRNAs significantly differently expressed between osteosarcoma cell lines and bones, $p \leq 0.05$.**
(XLS)

**Table S4 GeneGO enrichment of pathway maps for functional annotations of gene sets unique to cell line, gene set common for cell lines and clinical samples as well as gene set of 116 genes predicted to be targets of miRNAs in osteosarcoma.**
(XLS)

**Table S5 The Pearson's correlation (r) for each of the 67 miRNAs and its predicted mRNA targets across all the cell line samples.**
(XLS)

**Table S6 Pearson's correlation (*r*) between miRNAs and the putative target gene *PTEN*.**
(XLS)

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## Author Contributions

Conceived and designed the experiments: HMN LAMZ SHK OM. Performed the experiments: HMN SHK TB. Analyzed the data: HMN IHGØ LAMZ SHK. Contributed reagents/materials/analysis tools: MS HB AMCJ MLK OM LAMZ IHGØ SHK HMN. Wrote the paper: HMN LAMZ OM. Overall project planning: HB OM.

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